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Use of Short-Term Genotoxic Bioassays in the Evaluation of Unregulated Automobile Emissions

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USE OF SHORT-TERM GENOTOXIC BIOASSAYS IN THE EVALUATION OF UNREGULATED AUTOMOBILE EMISSIONS

by

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16. ABSTRACT

Exposure to fuels and fuel additives may occur through direct exposure to uncombusted fuel or fuel additive as well as to products of fuel/fuel-additive combustion. Presence of combustion products in ambient air is the major source of human exposure to motor vehicle fuels. The levels of several products of fuel combustion in ambient air (sulfur oxides, nitrogen oxides, fuel hydrocarbons and carbon monoxide) are currently regulated under the Clean Air Act Amendments [202(a)(4)] of 1977. These amendments also specify that new vehicles shall not be certified if they generate unregulated emissions which present a potential risk to human health. The objectives of this document are to review the data from selected short-term in vitro and in vivo bioassays to (a) determine if there is evidence suggesting potential human health risk either from uncombusted emissions or from emissions of combusted motor vehicle fuels or fuel additives, (b) identify the operational variables involved in generating products of concern for human health, (c) determine the probable nature of the health effects of concern, (d) estimate the ability of short-term tests to establish human risk estimates and (e) develop a short-term bioassay program to monitor the health effects of fuel/fuel additives and unregulated combustion emissions.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory, Research Triangle Park, NC and Cincinnati, OH conducts a coordinated environmental health research program in toxicology, epidemiology and clinical studies using human volunteer subjects. Wide ranges of pollutants known or suspected to cause health problems are studied. The research focuses on air pollutants, water pollutants, toxic substances, hazardous wastes, pesticides, and non-ionizing radiation. The laboratory participates in the development and revision of air and water quality criteria and health assessment documents on pollutants for which regulatory actions are being considered. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U.S. inhabitants.

F. Gordon Hueter, Ph.D.
Director
Health Effects Research Laboratory

PREFACE

The Environmental Protection Agency, under the Clean Air Act, is charged with the responsibility for regulating emissions from new motor vehicles and for the regulation of fuels and fuel-additives for motor vehicles and their emissions. The Clean Air Act specifically requires EPA to determine the potential public health effects of these emissions and specifies that these effects include carcinogenic and mutagenic effects.

Short-term genetic bioassays have now been widely employed by research scientists in government, industry, and independent research institutions to evaluate the mutagenic and potentially carcinogenic effects of motor vehicle emissions. The purpose of this document is primarily two-fold: (1) to summarize the current state-of-the-art related to applying short-term genetic bioassays to the evaluation of motor vehicle emissions and the assessment of potential human health effects, and (2) to recommend guidelines for evaluating unregulated combustion emissions from motor vehicle emissions using short-term genetic bioassays.

In preparing the final recommendations and guidelines for using short-term genetic bioassays, a scientific review and advisory workshop was convened. Scientists with expertise in this field from industry, government, and private research institutes were asked to scientifically review this document and specifically provide the recommendations which are reflected in the final section of this document.

David J. Brusick

Joellen Lewtas

ABSTRACT

Exposure to fuels and fuel additives may occur through direct contact with uncombusted fuel or fuel additives as well as products of fuel/fuel additive combustion. Presence of combustion products in ambient air is the major source of human exposure to motor vehicle fuels.

The levels of several products of fuel combustion in ambient air (nitrogen oxides, hydrocarbons and carbon monoxide) are currently regulated under the Clean Air Act. Amendments ([202(a)(4)] of 1977) also specify that new vehicles shall not be certified if they generate unregulated emissions which present a potential risk to human health. In addition, Section 211 of the Clean Air Act as amended in 1977 specifies that tests should be conducted to determine the mutagenic and carcinogenic effects (among other health effects) of automotive fuels and fuel additives and their emissions.

The objectives of this document are to review the data from selected short-term *in vitro* and *in vivo* bioassays to (a) determine if there is evidence suggesting potential human health risk from either uncombusted emissions or from emissions of combusted motor vehicle fuels or fuel additives, (b) identify the operational variables involved in generating products of concern for human health, (c) determine the probable nature of the health effects of concern, (d) estimate the ability of short-term tests to establish the human risk estimates, and (e) develop a short-term bioassay program to monitor the health effects of fuel/fuel additives and unregulated combustion emissions.

Meeting these objectives will involve analysis of each of the following areas:

1. Present state-of-the-art of short-term genetic test procedures and their applicability to the evaluation of fuels/fuel additives and combustion products.
2. Feasibility of developing risk estimates for human health effects from short-term bioassay data.
3. Review of short-term test data as they relate to motor vehicle fuels/fuel additives and unregulated combustion emissions.
4. Preparation of recommendations for the assessment of biological activity in fuels/fuel additives and combustion emissions using short-term bioassays.
5. Development of short-term screening strategy for application to ongoing assessment of fuels/fuel additives or combustion emissions from new sources.

This report was submitted in fulfillment of EPA Contract No. 68-02-3682, Technical Directive No. 008, by Litton Bionetics, Inc., under the sponsorship of the U.S. Environmental Protection Agency.

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LEGISLATIVE BACKGROUND FOR REGISTRATION OF FUELS AND FUEL ADDITIVES

Section 211 of the Clean Air Act as amended in 1977, charges the Administrator of the Environmental Protection Agency (EPA) with responsibility for regulation of fuel and fuel additives. Registration is required by the Agency of fuels and fuel additives prior to sale or introduction into commerce and requires the manufacturer of a fuel or fuel additive to:

- Conduct tests to determine potential public health effects of such fuel or additives (including, but not limited to, carcinogenic, teratogenic, or mutagenic effects); and
- Furnish the description of any analytical techniques that can be used to detect and measure any additive in such fuel, the recommended range of concentration of such additive, and the recommended purpose in use of such additives, and such other information as is reasonable and necessary to determine the emissions resulting from the use of the fuel or additives contained in such fuel, the effect of such fuel or additives on the emission control performance of any vehicle or vehicle engine, or the extent to which emissions affect the public health or welfare, with respect to each fuel or fuel additive which is registered on the date of promulgation of such regulations and with respect to each fuel or fuel additive for which an application for registration is filed thereafter.

In addition, manufacturers would be required to inform the Agency of mechanisms of action of additives, reactions between fuels and their additives, effects of the additives on emissions, identification and measurement of emission products, and effects of the emission products on health, welfare, and emission control devices.

Additive manufacturers would be required to furnish additional information about the chemical composition and structure of additives and methods of analysis, any impurities, and sales volumes. Fuel manufacturers would be required to furnish additional information on such properties as hydrocarbon composition, polynuclear organic materials, sulfur, trace elements, vapor pressure, distillation temperatures, and octane numbers, including methods of analysis for each of these, information on variation by geographic region, and production volume.

The registration of fuels and fuel additives is provided for in 46 CFR Part 79, A Revision of 40 CFR Part 79, (Federal Register, Volume 39, No. 46 (March 7, 1974). As revised, Part 79 designates certain fuels and additives as requiring registration: namely, motor vehicle gasoline and motor vehicle diesel fuels, and/or in motor vehicle crank case oil. These substances are further identified and designated in paragraphs 79.31, 79.32, 79.33 of Part 79 (46 CFR 79).

INTRODUCTION

The assessment of damage to the DNA molecule (genotoxic activity) by uncombusted fuels used in motor vehicles such as diesel fuel, unleaded gasoline, kerosene, ethanol, and methanol have not revealed evidence of significant genotoxicity (Lebowitz, 1978; ICPEMC, 1983). Emission products from the complete or partial combustion of fuels such as diesel fuel or gasoline, on the other hand, are associated with genotoxic activity, and a major portion of the genotoxicity is found in the solid phase (particulate) fraction as mutagenic and cell transforming organic compounds condensed onto the carbonaceous core of the exhaust particle (Vuk et al., 1976). Atmospheric parameters, such as humidity, temperature and UV irradiation existing at the time of collection, greatly influence the level and biological activity of organics bound to the particle core. Exhaust particles are one of many types of particles found as pollutants in ambient air. The current particle load for medium to large cities is approximately 100 $\mu g/m^3$ (Cuddihy et al., 1981); it is believed that these particles represent a major category of unregulated air pollution affecting human health.

Air pollutants include both gaseous and solid phases. Gaseous phase pollutants (i.e., carbon monoxide, ozone, nitrogen oxides and sulfur oxides) are recognized human health hazards and fall under regulatory standards of the Clean Air Act. The solid phase contains numerous organic compounds, polycyclic aromatic hydrocarbons (PAHs), and is suspected of contributing to the chronic toxicity (particularly lung effects) associated with long-term exposure to air pollution (i.e., the "urban factor") (Higginson et al., 1977; Higgins, 1977; Pike et al., 1975). Although the organic compounds associated with solid phase are not regulated, recent epidemiological evidence indicates that between 11 and 21 percent of human lung cancer may be caused by air pollution (Karch and Schneiderman, 1981).

Motor vehicle emissions are a major source of air pollution of both the gaseous and solid types (Table 1). The specific contribution of motor vehicle emissions to ambient pollution or to its associated health effects cannot be quantified, but based on the present understanding of the physical, chemical, and biological effects of the solid-phase emissions that are found in ambient air (Table 2), motor vehicle emissions might well contribute a disproportionate amount of the potential mutagenic and carcinogenic pollutants.

The possibility of human health risks from motor vehicle emissions has been a matter of concern for many years, and there has been substantial research into the effects of emissions from both gasoline spark ignition and diesel engines (National Research Council, 1981; USEPA, 1981). Even with this data base, the extent to which motor vehicle emissions contribute to human health problems is far from understood. Investigations have shown that complex interactions between fuel (and its additives) and gases in the combustion chamber, environmental exposure conditions and biology of the target species prevent most conventional hazard assessment methods to be applied for a reliable assessment. The multiplicity of complex interactions responsible for the difficulties in hazard assessment are shown in Figure 1. Each box in the figure represents a separate investigational effort that eventually must be integrated into the final human risk analysis. Some of the parameters identified in Figure 1 can be studied with current state-of-the-art techniques whereas others will require new test methods as well as novel approaches to data analysis. The suitability of short-term tests to evaluate the many facets of an emission's toxicity are listed in Figure 1 and summarized in Table 3.

Some of the important issues which have been addressed during the past several years of research are:

1. The chemical and physical characteristics of motor vehicle emissions and information on how atmospheric conditions alter those characteristics.

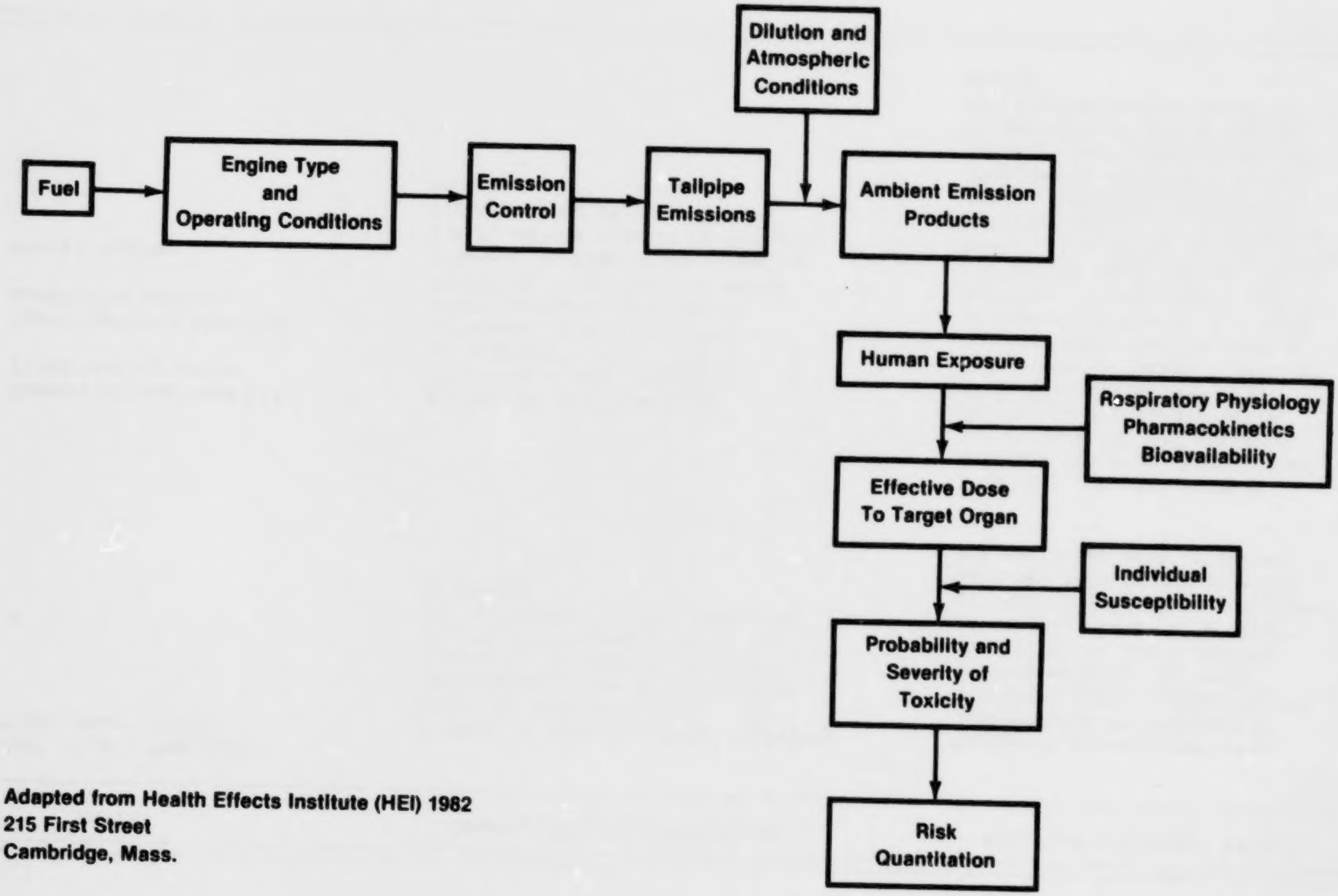
2. The influences of controllable parameters of fuel source, fuel additives and engine-running conditions on the physico-chemical and biological activities of emission products.

TABLE 1. ESTIMATED EMISSION PRODUCTS FROM VEHICLES IN 1981 (USEPA, 1981)

Pollutant	Thousands of Tons/yr	
	Gasoline	Diesel
Particles	5,650	774
Sulfur oxides	242	461
Nitrogen oxides	5,199	4,026
Free hydrocarbons	6,863	679
Carbon monoxide	73,839	1,629

TABLE 2. PARTICLES IN AMBIENT AIR

Sources	Chemical/Physical Properties	Reported Biological Effects
• Coal flyash from coal-fired power plants	A range of particle sizes including those of respirable size. The carbonaceous core contains bound inorganic and organic materials. The level of bound organics is low and generally less than 1.0% by weight.	Mutagenic activity has been demonstrated in prokaryotic microbial assays using organic solvent extracts of large quantities of stack-sampled particles. Level of biological activity is highly dependent upon the temperature at which the particles were collected.
• Commercial and home oil-fired heating units • Spark ignition gasoline combustion engines • Diesel engines	A range of particle sizes including a high proportion of respirable size. The carbonaceous core contains bound inorganic and organic materials. The level of bound organics is high often exceeding 10% by weight especially for oil-fired heating units and diesel engines.	Mutagenic and transforming activity has been demonstrated for organic solvent extracts *in vitro* using target cells. Organic extracts have also been shown to induce tumors in rodents under specific routes of administration (i.e., skin painting, intratracheal instillation and subcutaneous injection). The levels of biological activity appear to vary according to combustion efficiency, atmospheric conditions during cooling, fuel composition, and in the case of mobile sources, the running conditions of the engine.


Fuel
Engine Type and Operating Conditions
Emission Control
Tailpipe Emissions
Dilution and Atmospheric Conditions
Ambient Emission Products
Human Exposure
Respiratory Physiology Pharmacokinetics Bioavailability
Effective Dose To Target Organ
Individual Susceptibility
Probability and Severity of Toxicity
Risk Quantitation

Adapted from Health Effects Institute (HEI) 1982
215 First Street
Cambridge, Mass.

Figure 1. Automobile emissions and human health.

TABLE 3. USE OF SHORT-TERM TESTS TO INVESTIGATE THE SPECIFIC AREAS OF INTERESTS DEFINED IN FIGURE 1

Critical Parameters Identified in Figure 1	Application of Short-Term Test Methods
Fuel variations*	A single test engine can be run on different fuels for the minimum time sufficient to collect samples for one or two short-term tests. Multiple samples of the emissions can be compared for biological activity in a single assay simultaneously under the same test conditions. Using this approach the only variables which might give rise to differences in biological activity would be the fuels or fuel additives. If changes were observed, these could be tied to changes in the chemical composition of the collected emission.
Engine operating conditions*	Short-term tests could be applied as described above but the fuel source would be held constant and the engine or engine operating conditions would be varied.
Emission control*	Emission samples from uncontrolled and controlled engines could be collected and compared directly for different activities.
Tail pipe emissions	Small samples can be collected directly off the tailpipe, cooled, extracted and analyzed.
Atmospheric conditions and dilution effects	Short-term tests cannot directly provide answers to the dynamics of these processes but can be used to compare emission samples collected under various real or simulated atmospheric and/or dilution conditions.
Ambient emissions	Ambient air samples of sufficient size for short-term tests can be collected however the proportion of the sample or the measured biological activity contributed by vehicle emissions must be estimated from source tracer compounds (e.g., lead) using apportionment methodologies.

TABLE 3 (Continued)

Critical Parameters Identified in Figure 1	Application of Short-Term Test Methods
Secondary emission products	Secondary emission products, formed after atmospheric reactions (eg. peroxyacetylnitrate) can be studied in smog chambers. Short-term tests have been applied to samples taken from smog chambers and methods are being developed to directly bioassay gaseous mixtures of such products.
Human Exposure	Some short-term in vitro tests utilize human derived target cells. Short-term tests are also being developed for application to in vivo human exposures.
Respiratory physiology and pharmacokinetics	Short-term tests are not currently applicable.
Effective dose	Short-term tests are not currently applicable, although studies of phagocytosis and metabolic processing of organic laden particles can be studied in a limited number of the short-term tests. The exposure, however, is still in vitro. Methods by which relevant target cells can be collected from animals exposed in vivo and then subjected to in vitro analysis should be available in the near future. Such tests may provide an effective probe to answer this question.
Individual susceptibility	Short-term tests are not currently applicable.
Probability and severity of risk	Short-term tests can be applied to either qualitative hazard determinations or quantitative risk estimates.

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*The amount of emission sample available from an engine running for a reasonable period would not be sufficient to make comparisons over a dose response curve using conventional animal tests for mutation or tumor induction. The amount of sample from an engine with a pollution control device would be less than an uncontrolled engine making comparisons in animals even less likely.

3. The probable types of human health effects associated with exposure to motor vehicle emissions.

4. The specific chemical components of motor vehicle emissions which are most likely responsible for the health effect of concern.

5. Assessment of the potential for human risk will be derived from both *in vitro* and *in vivo* studies.

The objective of this report is to summarize the current status of fuel/fuel additive research using short-term tests for genotoxicity and to develop an approach for the application of these tests to the development of regulatory strategy. Short-term genetic tests are believed to be indicators of carcinogenic and mutagenic hazard for humans on the basis of extensive studies with pure chemicals; thus, such tests should be used in addressing the concern for carcinogenic effects. The use of the term test is not meant to imply that these methods are standardized or uniformly reproducible. Short-term methods are continually undergoing validation, refinement and reinterpretation.

The final report will be divided into seven sections. The first section will summarize the current state-of-the-art of short-term genotoxicity testing as it applies to studies of motor vehicle emissions. The methods used in studies of vehicle emissions will be described, and their strengths and limitations in the context of the more conventional long-term toxicology techniques will be reviewed.

The second section of the report will be a review of the application of short-term bioassays in assessing human risk. Human risk analyses are usually based upon epidemiology studies or extensive animal modeling. However, automobile emissions do not lend themselves to the current animal model techniques used for making risk assessments, nor are available human epidemiology studies adequate to develop a reliable risk estimate. Consequently, the initial attempts at risk analysis for motor vehicle exhaust emissions have relied heavily on the results of short-term tests.

The third section of the report will consist of a review of the research efforts directed toward the evaluation of motor vehicle emissions using short-term tests. Short-term bioassays have been used extensively to evaluate the solid phase pollutants (particles) and their extracts by determining the biological properties of whole exhaust and collected particles. The flexibility and adaptability of *in vitro* assays were also evident from data involving assessment of extracts or leachates from collected particles and their subfractions. These studies have resulted in the identification of chemicals or chemical classes associated with the biologically active samples.

Section four of the report will attempt to delineate critical unresolved issues related to possible human health risk. This section will also include recommendations for possible additional research that would help fill existing data gaps.

Section five will contain recommendations for guidelines that could be applied to the evaluation of "unregulated" vehicle emissions on a continuing basis.

Section six will outline the types of tests that could be included in a testing program for certification of motor vehicles on the basis of genotoxic predictive tests.

The final section will be a summary of the entire document with a statement of principles concerning the current level of understanding of the genotoxicity of automobile emissions.

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PART I

SECTION 1

A SUMMARY OF THE CURRENT STATE-OF-THE-ART OF GENOTOXICITY TESTING APPLICABLE TO STUDIES OF MOTOR VEHICLE EMISSIONS

GENERAL APPLICATION

Short-term genotoxicity testing fills the rather specific role in hazard assessment as a cost-effective predictor of nonreversible toxicity, namely cancer and genetic disease (Ames, 1979; Hollstein *et al*., 1979; Brusick, 1978). Utility of these tests is dependent upon their acceptance as reliable models for actual *in vivo* responses. Consequently, many of them have been subjected to correlation investigations to determine their quantitative predictive coefficients for cancer and/or heritable genetic disease in animals (Purchase, 1982). Details of these comparative studies as they relate to specific bioassays will be discussed in Sections 2 and 3. While the assays are generally considered reliable indicators of carcinogens, false positive and negative responses have been generated.

Bioassays which qualify as short-term predictive tests include submammalian, *in vitro* mammalian and *in vivo* techniques to identify which chemicals induce alterations in DNA or chromosomes or produce cell transformation. The most widely used tests are listed in Table 4. They are identified by their applicability to either carcinogenicity and/or mutagenicity prediction.

A general consensus has evolved which describes cancer as a multi-stage process involving a variety of events including initiation, repair, fixation, promotion and proliferation (Figure 2) (Weisburger and Williams, 1981). Because genotoxic steps are implicated early in this process, their detection has assumed the status of presumptive carcinogen identification. This assumption is not without some controversy and a good objective review of the benefits and problems associated with application of the short-term technology to carcinogen screening is given in ICPEMC Committee 2 Report, 1982.

The range of bioassays which fall into the short-term category is very broad and the number of tests is quite large. The EPA Gene-Tox program has reviewed the published literature for approximately 35 separate tests (Waters and Auletta, 1981) and prepared a series of papers which evaluate the performance of each test. Ultimately this program will attempt to define the applicability of specific short-term tests as carcinogen and mutagen predictors and will recommend specific test batteries. Not all

TABLE 4. A SUMMARY OF THE POSSIBLE ROLES FOR SELECTED GENETIC TESTS IN CHEMICAL HAZARD ASSESSMENT

General Assay Type	Primary Roles	
	ICP	IMP
Microbial Assays		
Ames Reverse Mutation Test	++	+
Reverse Mutation in *E. coli* WP_2 and Related Strains	+	+
Bacterial DNA Repair Tests	+	NA
Yeast Mutation Tests	+	++
Yeast Mitotic Recombination	+	NA
In Vitro Mammalian Cell Assays		
Mouse Lymphoma Assay (TK)	+	++
CHO or V79 Mutation Assays (HGPRT)	+	++
Unscheduled DNA Synthesis (UDS)	++	NA
Chromosome Aberrations	+	++
Sister Chromatid Exchange (SCE)	++	NA
Cell Transformation	++	NA
In Vivo Mammalian Assays		
SCE	+	NA
Dominant Lethal Assay	NA	++
Cytogenetic Analysis (aberrations)	+	++
Micronucleus Assay	+	+
Spermhead Abnormality Assay	NA	(+)
Heritable Translocation Assay in Mice	NA	+
Specific Locus Assay in Mice	NA	++
DNA Adduct Formation	+	(+)
UDS Assays	+	(+)
In Vivo Submammalian Assays		
Drosophila Assays	+	++
Plant Cytogenetics	NA	(+)

ICP = Identifies Mammalian Carcinogenic Potential
IMP = Identifies Mammalian Mutagenic Potential
+ = Applicable
++ = Greater Applicability for this Role
NA = Not Applicable
(+) = Possible Application Under Limited Conditions

Step	Comment
Initiating Event	This is probably the initial event and is a mutation or DNA lesion affecting a particular gene or set of genes. This step is irreversible but requires subsequent steps for expression.
Fixation	The genetic lesion requires specific metabolic processes to occur and/or cell division to fix the lesion into the genome of the cell.
Promotion/Enhancement	It is assumed that specific physiological or biochemical phenomena are required to facilitate the expression of the initiating event. Other chemicals, such as phorbol esters, hydrocarbons, phenobarbital, smoke condensate, etc., have been implicated in the promotion and enhancement of cell transformation. To a certain extent these processes appear reversible.
Expression of Cell Transformation	The cell acquires a new set of genetic biochemical and immunological properties. The properties give rise to the tumor properties of the particular neoplasm. All progeny cells of the tumor acquire these properties fully expressed and do not go through the transformation process.
(Evades Immune Surveillance System of Host)	
Tumor	When a sizable population of transformed cells is formed by rapid proliferation, the neoplastic disease is identified clinically as a tumor mass.

Figure 2. Hypothetical steps involved in oncogenic transformation.

of these tests, however, will be applicable to the evaluation of motor vehicle emissions. In order for a bioassay, even highly predictive assays, to be suitable for this type of investigation it should have the following characteristics:

1. The method should be sufficiently flexible to test both particles (1-10 μm size) as well as aqueous- or organic-solvent-soluble complex mixtures that are extractable from the particle core.

2. The technique should have intrinsic metabolic activation/detoxification capabilities or be compatible with a cell-free exogenously supplied metabolic activation system.

3. The test should be capable of generating a reproducible dose-related response following exposure to genotoxic agents and produce data that can be evaluated by conventional statistical procedures.

4. The method at least should be responsive to PAH carcinogens since this class of agents predominates in emissions.

5. The test should be amenable to assessment of small samples (10-500 mg quantities).

Only a fraction of the total assay of genetic bioassays satisfies all five criteria. The last two criteria are not only very important but are also responsible for ruling out many otherwise suitable test methods.

SPECIFIC LIMITATIONS TO SHORT-TERM TEST APPLICATION

Exhaust particles are generally less than 1 micron in diameter and easily transported to the deep lung in humans (Huisingh *et al*., 1978). Because of the presence of organic carcinogens on the particles, solid-phase pollutants appear to be the most likely lung carcinogen candidates. The finding that essentially all relevant genotoxic effects of gasoline or diesel exhaust emissions were associated with the particle phase has significantly affected the applicability of short-term tests to studies of exhaust emissions (McClellan *et al*., 1981). One limitation of *in vitro* tests is inability to conduct reliable aerosol exposures of biphasic emissions. Some success with vapor-phase exposures in microbial tests have been reported but, in general, mammalian cell *in vitro* tests have not been adopted to this exposure route. Genotoxic chemicals are produced during fuel combustion but, shortly after the emissions leave the tailpipe, a significant portion of these agents are condensed onto the carbonaceous particle core. To a certain extent this lessens the requirement for testing using only whole-exhaust emissions.

In addition to their limitations with respect to certain routes of exposure, short-term tests have other characteristics which reduce their applicability to some important emissions research problem areas. For instance, one most critical issue involved in the risk analysis question is that of target-organ dosimetry by the biologically active component of the emission. Understanding this part of the risk process for air pollutants must take into account data on target organ exposure to particles, deposition sites of particles in the respiratory system, and clearance kinetics. Also of critical importance to the assessment of possible health risks are in vivo desorption rates for bound, toxic organic and inorganic materials and transport of biologically active substances to susceptible target cells. Short-term, especially in vitro, tests cannot be applied easily to the resolution of these problems, with the possible exception of intracellular transport of specific organic chemicals. Several other available techniques show cellular absorption of particles, but these types of short-term studies on whole exhaust are severely limited.

SPECIAL APPLICATIONS

The inability to study relevant exposure conditions using in vitro tests does not completely exclude the use of short-term tests in target organ dosimetry. It appears possible to screen for genotoxic effects in respiratory tract target cells following in vivo inhalation or instillation exposures in hamsters (Guerrero and Rounds, 1981). However, at the present time, these methods are developmental and little data from motor vehicle emissions are available. Exposure to genotoxic fractions in these bioassays is detected via DNA binding, SCE, or stimulation of DNA repair processes. These tests and their results are reviewed in Section 3. If these techniques can be developed further and validated, they would enhance considerably the opportunity to study target-cell dosimetry under well defined aerosol-exposure conditions.

Interest has also been raised about the possible enhancement of the carcinogenicity effects caused by motor vehicle emissions as a result of interactions between the PAHs bound to the solid phase and some of the irritating gases present in exhaust emissions. Reports from Ziem et al. (1982), and Laskin et al. (1970), indicated that SO_2, NO_2 and O_3 vapors enhance the carcinogenic effects of known carcinogens. If these interactions are important for expression of the carcinogenicity of exhaust PAHs, in vitro experiments would not give accurate estimates of the associated hazard. However, results from ongoing in vivo studies of such interactions might reveal their significance in risk assessment. Heinrich et al. (1982), for example, has shown that tumor rates in Syrian hamsters exposed to diethylnitrosamine are increased following exposure to particle-free diesel exhaust. Short-term tests addressing this phenomenon have not been reported.

Metabolism, especially of PAHs, plays a major role in toxicity extrapolation across phylogenetic barriers. The ultimate biological activity of a chemical in vivo is a function of the three processes of activation, deactivation occurring together and reactivation. The balance of these processes is a major factor in determining the carcinogenic potential of a chemical. Integration of these functions under the conditions of in vitro metabolism used in short-term bioassays might well vary considerably with the metabolic processes in vivo. Well defined metabolic studies with complex mixtures are difficult because of the need to selectively label specific compounds among the hundreds present.

SUMMARY

In summary, many characteristics of short-term genotoxicity tests appear ideally suited to the analysis of automobile exhaust emissions. In fact, much of the information available on the biological properties and possible health risks associated with the particulate emissions has been accumulated largely using short-term test data. Certainly the opportunity to conduct multi-dose experiments on the relatively small quantities of particles collected from exhaust emissions was one of the significant breakthroughs in the evaluation of motor vehicle emissions. Many of the attributes of short-term tests are important to the application of these tests and are summarized in Table 5. The importance of the ability to conduct assays using very small samples cannot be overemphasized since spark-ignition gasoline engines equipped with catalytic converters produce very low levels of particulate; thus, significant comparisons of these samples with other emissions would be impossible without short-term assays.

TABLE 5. CHARACTERISTICS OF SHORT-TERM BIOASSAYS WHICH FACILITATE THEIR APPLICATION TO HEALTH EFFECTS EVALUATION OF AUTOMOBILE EMISSIONS

Characteristic	Application to Emission Testing	Comments
Sample size required to conduct the test is small.	This characteristic permits the evaluation of particulate samples collected on filters under simulated ambient conditions. Most animal model studies would require substantially more collected particle samples or could be conducted only on whole or scrubbed exhaust containing both gaseous and solid phases.	Limitations of animal studies to whole exhaust is technically difficult because of the levels of toxic vapors present at high exposure conditions. In order to enhance exposure to the presumed carcinogens, exposure to particulates would be necessary.
Biodirected fractionation of complex mixtures	Some of the *in vitro* or submammalian short-term tests can be used to direct sample fractionation and chemical characterization. This is possible because of the high degree of test sensitivity and the ability to perform tests on very small samples of fractionated test sample. Extracts and/or leachates of particles collected from emissions have been fractionated to identify which fractions retain genotoxic activity and which do not.	It will be necessary eventually to identify specific chemicals (or chemical classes) of concern in the collected emissions. Once these agents are identified and linked to the specific health effects, permissible levels can be established and emissions from new fuels, fuel additives or engines can be evaluated rapidly.
Test methods are amenable to quantitation of test results which involves dose response	To develop the types of information which might be applicable for hazard assessment, data from comparative studies will be required. Accurate comparisons can only be generated from dose-response curves, especially from replicate studies.	Comparative data (e.g., two types of fuels combusted in one type of engine) cannot be developed readily using animal models because of technical facilities limitations. Yet without direct comparisons, risk analysis cannot be formulated.

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PART I

SECTION 2

THE CURRENT STATE-OF-THE-ART IN APPLICATION OF SHORT-TERM BIOASSAYS TO AN ASSESSMENT OF HUMAN CANCER AND MUTATION RISK

Genotoxic testing can be used to estimate the qualitative risk potential of chemicals as mutagens and carcinogens. Approaches to evaluate the two phenonema cannot be identical because of the mechanistic and methodological reasons cited in Table 6. However, since numerous aspects of the application of short-term tests to assessing the human risk of cancer and mutation are similar, these dysfunctions generally will be combined for the purposes of this discussion.

Mechanistically, chemicals which alter the structure or function of DNA are capable of inducing cancer or genetic dysfunction in humans in that lesions which give rise to mutations or to the initiating lesions of cancer result from specific chemical-DNA interactions described in Table 7. The essential questions, then, are those of exposure levels of the appropriate target cells (somatic or germinal), intrinsic potency of the genotoxic material, and bioavailability of the carcinogenic moiety to susceptible target cells of the appropriate species at sufficient concentrations to induce genetic damage (Figure 3). The genotoxic risk to somatic and germ cells posed by effects of the same chemical can differ greatly, somatic cells appear to be more susceptible for several reasons listed in Table 8.

In order to approach risk assessment from an operationally feasible point-of-view, the process needs to be separated into two functions (a) detection and (b) quantification.

DETECTION OF HAZARD

Qualitative determination of hazard is based on observation of (a) increased cancer or genetic effects in human populations exposed to a particular substance, (b) experimental data from animal model systems in which the tumor incidence or mutation rates are increased as the result of exposure to genotoxic chemicals, or (c) positive results from short-term predictive tests which rely upon mechanistic linkages and correlation coefficients to signify that such chemicals, when identified, may be shown in subsequent studies as carcinogenic/mutagenic hazards.

TABLE 6. COMPARISONS OF THE APPROACHES USED IN PREDICTIVE SCREENING FOR CARCINOGENS AND MUTAGENS

Parameter	Carcinogenesis Prediction	Mutagenesis Prediction
Animal Test Data Reference Standard	Consists of a rather large body of data (300-500 experimental carcinogens) in rodents with a diverse group of chemical classes.	Consists of a small body of data (50-100 germ cell mutagens at best) in rodents with a relatively narrow spectrum of chemical classes (largely direct-acting alkylating agents).
Human Epidemiology	Substantial body of data available. In approximately twenty cases, direct cause/effect relationships have been established between chemical exposure and cancer.	Few epidemiology studies. Evidence for cause/effect relationship between specific agent and human genetic effects is virtually nonexistent for chemical mutagens.
Mechanism of Induction	Presumably initiated by genotoxic damage produced by exposure to carcinogenic agent; however, tumor response can be significantly modified by secondary phenomena and are often the consequence of an exposure to a second, nongenotoxic chemical (promoters, cocarcinogens).	Mechanism involves altered DNA base sequence or loss of chromosome integrity in target cell. There is no evidence for modulation of mutation in germ cells by nonmutagenic chemicals.
Risk Extrapolation Methods	Many mathematical models available. The methods vary from linear nonthreshold to nonlinear threshold models depending on the likely mechanism involved in tumor induction.	Current approach seems to be limited only to a linear nonthreshold mathematical treatment of the data. This is a result of the single hit theory of mutation induction.

TABLE 7. MAJOR DNA LESIONS PRODUCED BY CHEMICAL INTERACTION AND THEIR GENOTOXIC CONSEQUENCES

Primary Lesion	Description	Consequence
Alkylation	Covalent adduct formed involving the genotoxic agent and a DNA base or phosphodiester bridge.	Alteration of base-pairing loss of the base, stimulation of error-prone repair.
Intercalation	Noncovalent stacking of the genotoxic agent between adjacent base pairs in the DNA helix.	Alteration of DNA transcription, replication or repair.
Cross-Linkage	Formation of two covalent bonds between bases within (intrastrand) or between (interstrand) DNA strands.	Dimer formations, alteration of replication.
Breakage	Scission of either a single or both strands of the DNA helix	DNA rearrangements forming chromosome aberrations after mitotic cell division.

N_1 (Intrinsic Genetic Potential of Chemical)	X	N_2 (Opportunity for Hits on Target Molecule at Normal Exposure Levels)	= R (Risk)

- **DETECTION OF HAZARD**

  N_1 can be detected using various assay systems on a qualitative basis; however, potency is difficult to measure except on a relative scale. Extrapolation of qualitative results from species to species also introduces uncertainty into the equation.

- **DETERMINATION OF EXPOSURE**

  N_2 cannot usually be quantitatively measured at the low environmental levels normally encountered. Even qualitative evidence for target molecule hit is difficult at low levels because of the magnitude of the populations that would be required. Short-term tests may be able to approach the determination of N_2 because of their increased sensitivity and resolving power.

- **RISKS CALCULATION**

  R can be roughly estimated at high dose levels using some type of N_1 and N_2 values. The meaning of R at high N_1 and N_2 levels is difficult to interpret and may not be directly extrapolatable to low levels.

Figure 3. Considerations involved in quantitative risk assessment.

TABLE 8. A COMPARISON OF RISK ASSESSMENT FOR SOMATIC AND GERM CELLS *IN VITRO*

Comparison Parameter	Somatic Cells	Germ Cells
Selective Pressure on New Mutants	Slight, since cells encounter only mitotic division.	High, since meiotic cell cell division acts to screen out specific types of mutations and chromosome effects.
Barriers to Mutagenic Agents	Few barriers if agent is distributed systemically.	Barriers similar to those for somatic cells plus blood-gonadal barriers.
Sex Differences	Minimal; some influence of hormones on metabolism in specific organs.	Significant because of the different processes involved in gametogenesis between male and female mammals.
Ease of Detection of Induced Effects	Each dosed animal represents a population of exposed cells.	Analysis generally requires large animal populations since each offspring represents a single exposed gamete analyzed.
Size of Exposed Sample that can be Scored	Extremely large population, generally 10^5 to 10^7 surviving cells scored.	Generally small sample sizes; less than 10^5 animals in all cases.
Type of Genetic Lesion Detected	Chromosome aberrations and single gene mutations both easily detected.	Chromosome aberrations typically scored. Point mutations require extensive studies to detect with reliability.

QUANTIFICATION OF HAZARD

This phase of the risk analysis process involves an estimation of the incidence of adverse effects in humans under anticipated exposure conditions encountered by the population at risk. The data providing the foundation for this part of the risk analysis are generally weak because analysis is based on unverifiable assumptions and extrapolations of high dose responses to estimates of low dose response and often requires extrapolation across vast phylogenic gaps. The high-to-low-dose extrapolations evoke considerable uncertainty and are often the subject of scientific controversy (Munro and Krewski, 1980).

APPLICATION OF CONVENTIONAL RISK ASSESSMENT METHODS TO AUTOMOBILE EMISSIONS

Epidemiological evidence in humans provides the strongest possible risk assessment information when studies show a positive cause-effect relationship. All chemicals currently recognized as human carcinogens have been identified as such by epidemiological studies (Bartsch *et al.*, 1982). Both the hazard detection and quantification information come from the relevant target species. Unfortunately, most risk estimates have not been derived from human epidemiological data because of the limited nature of valid human retrospective studies. One human retrospective epidemiological study of motor vehicle emissions employed bus garage workers (men 45 to 64 years old employed by the London Transport Authority during the years 1950 to 1974) who showed no carcinogenic effects from long-term exposure to diesel exhaust emissions (National Research Council, 1981). The cohort consisted of bus garage engineers, bus drivers, bus conductors, central work engineers, motormen, and guards. Calculations made to estimate the possible magnitude of an undetected effect showed that an undetected incidence of lung cancer in unexposed workers could not be excluded by the study.

Using the London bus garage study, an estimate of lung cancer risk under ambient conditions was calculated to be a 0.05 percent proportional increase in lung cancer incidence per unit of exposure to diesel engine emissions (1 unit = 1 μg inhaled particles/m^3 per year). However, this study was limited in many respects and cannot be considered a completely valid risk assessment for exposures to ambient motor vehicle emissions.

Attempts to apply animal models to the risk assessment of motor vehicle emissions have likewise not been very successful for the following reasons:

1. The classes of chemicals identified in diesel or gasoline automobile exhaust emissions (PAHs) are relatively inactive in the typical rodent model systems used (mouse and rat). Inhalation studies using whole diesel exhaust or pure aerosolized PAH such as benz(a)pyrene (BaP) did not produce cancer in mice or rats (Battelle-Northwest Laboratories, 1982; Heinrich *et al.*, 1982; GM Research Center, 1982), although lung tumors have been induced in hamsters using extremely high levels of a pure carcinogen (Thyssen *et al.*, 1981). Solvent extracts of gasoline and diesel exhaust particulates have been shown to produce weak tumorigenicity when administered in skin painting and subcutaneous injection studies in mice, in intratracheal instillation in hamsters and in two stage promotion systems in mice (Kotin *et al.*, 1954; Wynder *et al.*, 1962; Mohr *et al.*, 1976; Brune *et al.*, 1978; Misfeld, 1980, Slaga *et al.*, 1980).

2. The levels of particles containing bound carcinogens in ambient or diluted exhaust emissions are so low that calculations based on the benz(a)pyrene content of diesel exhaust indicates that no more than 1 µg of BaP will be deposited in the lungs of hamsters exposed for two years to diluted exhaust under experimental test conditions. This level is considerably lower than the 10 mg/m^3 of pure BaP required to induce lung tumors in hamsters exposed by inhalation and almost precludes the observation of carcinogenesis activity of exhaust emissions in reasonably sized animal experiments.

3. Experimental inhalation studies, which represent the most relevant exposure route for humans, are complicated by the presence of both irritant (NO_2/SO_2) and lethal (CO) gases. To obtain high levels of emission particles in the inhalation chambers without a high level of mortality among the test animals, the exhaust emissions from either gasoline or diesel engines must be scrubbed to remove CO, NO_2 and SO_2 which is likely to result in the alteration of the composition of organic chemicals bound to the particles.

Thus, the general consensus is that conventional lifetime inhalation studies of diluted automobile exhaust emissions will not provide the necessary data to develop an accurate estimate of human risk. Other animal models such as skin painting in SENCAR mice and intratracheal instillation in hamsters may provide data of limited utility, but these tests suffer from limitations in relevant route of exposure and test system refractibility.

APPLICATION OF SHORT-TERM BIOASSAYS TO RISK ASSESSMENT OF AUTOMOBILE EMISSIONS

Because short-term, and especially *in vitro*, assays have been found to be readily applicable to an evaluation of solid phase emissions, much of the data predicting qualitative hazard has been derived from this type of research. Risk analysis must draw upon all available data. Quantitative estimates developed for automobile exhaust emissions by the Environmental Protection Agency (EPA) and the National Academy of Sciences (NAS) have relied heavily upon comparative data obtained from *in vitro* assays for genotoxicity and short-term animal tests (Albert *et al.*, 1982; National Research Council, 1981). Both approaches were based upon available short-term data and comparative analyses were made of laboratory and epidemiological data on diesel and gasoline engine exposure as well as other chemically related environmental exposures--coke-oven-emission, roofing-tar-emission and cigarette-smoke-condensate (CSC). Types of data integrated into the two risk analyses are summarized in Table 9. The NAS analysis excluded the Ames data primarily because the test results from bacteria studies are enhanced when compared to mammalian studies because of microbial nitroreductases. Mammalian cells are generally less responsive per unit dose because of reduced nitroreductase activity. The SCE data used in the EPA analysis were not available for inclusion in the NAS assessment.

The short-term data base for diesel exhaust emissions extends far beyond the bioassays listed in Table 9, but the information reported from these assays met the type of criteria required for biostatistical analysis.

TABLE 9. DATA SOURCES USED TO PREPARE LUNG CANCER RISK ASSESSMENTS FOR AUTOMOBILE EXHAUST EMISSIONS BY NAS AND EPA

	NAS[a]	EPA[b]
Bioassays Used		
• Reverse mutation in *Salmonella typhimurium*		✓
• Forward mutation at the TK locus in L5178Y mouse lymphoma cells	✓	✓
• Sister chromatid exchange (SCE) in Chinese hamster ovary cells		✓
• Enhancement of viral (SA7) transformation of Syrian hamster embryo cells	✓	✓
• SENCAR mouse skin tumor initiation	✓	✓
Related Environmental Exposures Used		
• Coke oven emissions	✓	✓
• Roofing tar emissions	✓	✓
• Cigarette smoke condensate (CSC)		✓
Emission Sources Compared		
• Nissan diesel (LD)[c]	✓	✓
• Volkswagen diesel (LD)	✓	✓
• Oldsmobile diesel (LD)	✓	✓
• Caterpillar diesel (HD)[d]	✓	✓
• Ford Mustang II gasoline		✓

[a] NAS = National Academy of Science (National Research Council, 1981)
[b] EPA = Environmental Protection Agency (Albert *et al*., 1982)
[c] LD = Light Duty Engine
[d] HD = Heavy Duty Engine

The reasons that the short-term data from the tests listed in Table 9 were amenable to use in risk estimation are:

1. Results were reproducible within a given bioassay.
2. Experiments provided dose response results spanning a broad range of concentrations.
3. Experimental results could be formatted in a manner amenable to statistical analysis.
4. Each bioassay was considered a valuable correlate with carcinogenicity or mutagenicity in mammals.
5. Each bioassay was suitable for analysis of other environmental emissions with related composition (roofing tar emissions and coke oven emissions).

Both the NAS and EPA risk estimates analyzed the short-term test results in terms of a linear non-threshold extrapolation model. The potency response of all sample emissions evaluated in the short-term bioassays was compared using the linear extrapolation modes. Relative potencies for gasoline and diesel emissions were then compared to those for coke oven, roofing tar and cigarette smoke condensate. Assuming comparability between relative potency for the diesel emissions and the model emissions (coke oven or roofing tar) a relative potency value for humans was constructed using the human lung cancer data from coke-oven workers and roofers.

The estimate for lung cancer risk from exposure to diesel-exhaust emissions was derived by determining the ratio of short-term test activities between the two emissions and extrapolating that ratio to the incidence of human lung cancers for the two exposures after adjusting for dose. The calculated risks are given in Table 10 and are quite similar even though the two groups of short-term bioassays studied were not identical. It is also of interest to note that the estimated risks determined by NAS and EPA were similar to the estimated lung cancer risk for individuals exposed to ambient diesel-exhaust emissions in the London bus garage workers study. However, the expression of NAS and EPA risks are in absolute risk while the London bus worker study gave proportional risks.

The EPA and NAS efforts may have established a precedent for the use of short-term test results in combination with epidemiological data for surrogate materials in estimating human risk, especially in cases of complex mixtures. An analysis of each risk estimate is provided in the primary reference document which outlines the limitations and assumptions of the two approaches. Questions of how to apply the risk estimates remain unanswered because the lung cancer estimates were made on the basis of organic solvent extracts of emissions particles. It is suspected that the bioavailabilities of organic carcinogens from diesel emissions, gasoline emissions, coke oven emissions, roofing-tar emissions and cigarette smoke condensates are significantly different. Some of the

TABLE 10. HUMAN LUNG CANCER RISK ESTIMATES FOR DIESEL AND GASOLINE-CATALYST PARTICULATE EMISSIONS BASED ON SHORT-TERM TEST DATA

Emission Source	Unit-Risk Estimates (Lifetime Risk/$\mu g/m^3$)	
	NAS[a,b]	EPA[c]
Nissan diesel	$0.2\text{-}0.6 \times 10^{-4}$	0.35×10^{-4}
Oldsmobile diesel	$0.1\text{-}0.24 \times 10^{-4}$	0.2×10^{-4}
Volkswagen diesel	$0.02\text{-}2.78 \times 10^{-4}$	0.23×10^{-4}
Caterpillar diesel	$0.01\text{-}0.25 \times 10^{-4}$	0.018×10^{-4}
Ford Mustang gasoline	--	0.60×10^{-4}

[a] NAS = National Academy of Science (National Research Council, 1981)
[b] A range of estimates was given for the NAS study. Both estimates are for emission particulates. Similar estimates made by the EPA based on total organics extractable from particles are five to ten times greater.
[c] EPA = Environmental Protection Agency (Albert *et al*., 1982)

active compounds in the mixtures are also different. Bioavailability, then, is a major undetermined variable in exposure to automobile emissions. Another important unanswered question is that of susceptible subpopulations (age, sex, disease state). Most, if not all, relevent human epidemiology data for the surrogate emissions were obtained from adult males under occupational exposure conditions whereas diesel emission risks will include people of both sexes and of all age ranges and health conditions.

The following list identifies areas of concern or uncertainity in the application of short-term test data to human risk analysis.

1. Are differences in identity and bioavailability of carcinogens from the test and surrogate emissions sufficient to affect the risk calculations?

2. Was the selected battery of short-term tests the most reliable for estimation of cancer risk?

3. Considering that cancer potency can be substantially modulated *in vivo* by promoting agents and cocarcinogens and that most short-term tests are refractory to these agents, is it possible that the risk estimates would change if promotion or cocarcinogenic activities were factored into the calculations?

4. How reliable is the relationship between genotoxicity and carcinogenicity?

There are no available data to apply to the answers to questions 1-3, but extensive studies have been conducted addressing question 4.

Overlapping mechanisms and correlation studies provide the rationale for the application of genotoxicity results to human risk analysis. Most reports evaluating the reliability of short-term test batteries as predictors of cancer in mammals indicate a high level of predictive power (85 to 95 percent). While there may be serious concerns for the uncertainity associated with the quantitative extrapolation of short-term test results to humans, on a qualitative basis (detection of hazard) short-term tests are as reliable, if not more reliable, than conventional animal models in human carcinogen detection (Table 11). Results from several short-term tests can be used as reasonably good predictors of heritable genetic risk in mammals (Table 12).

In summary, results from short-term tests for genotoxicity are amenable to use in making human risk estimates in situations where conventional animal modeling or human epidemiology results cannot be obtained. An evaluation of two approaches conducted by EPA and NAS show similar quantitative risk estimates for diesel exhaust emissions. These two evaluations are also within two orders of magnitude of a worst case estimate for lung cancer in humans derived from negative epidemiological data. Risk estimation for genetic effects cannot yet be accomplished using similar methods because (a) a different set of short-term bioassays are needed to predict germ cell damage (Table 12) and (b) there are no human risk estimates for genetic effects derived from epidemiology data on surrogate emissions.

TABLE 11. CHEMICALS EVALUATED AS CARCINOGENIC FOR HUMANS AND THEIR RESPONSES IN RODENT AND SHORT-TERM PREDICTIVE ASSAYS

Group 1 Chemicals[a]	Human	Mouse Bioassay	Rat Bioassay	Short-Term Bioassays[b]
4-Aminobiphenyl	+	+	+	+
Analgesic mixtures containing phenacitin	+	-	+	+
Arsenic and arsenic compounds	+	-	-	+
Asbestos	+	+	+	+
Azathioprine	+	(+)	(+)	+
Benzene	+	-	-	+
Benzidine	+	ND	+	+
N,N-Bis (2-chloroethyl-2) naphthylamine (chlornaphazine)	+	+	+	+
Bis(chloromethyl)ether and chloromethyl methyl ether	+	+	+	+
Certain combined lymphoma chemotherapy (MOPP)	+	ND	ND	+
Chlorambucil	+	+	+	+
Chromium and chromium compounds	+	-	+	+
Conjugated estrogens	+	ND	-	+
Cyclophosphamide	+	+	+	+
Diethylstilbestrol	+	+	+	+
Melphalan	+	+	+	+
Methoxysalen plus UV light (PUVA)	+	+	ND	+
Mustard Gas	+	+	ND	+
Myleran	+	(+)	-	+
2-Naphthylamine	+	+	-	+
Soot, tars and oils	+	+	-	+
Treosulphan	+	ND	ND	+
Vinyl chloride	+	+	+	+

[a] Not included are manufacturing or mining processes which do not involve discrete chemical entities.

[b] Bioassays include the Ames test, a cell transformation test and tests for chromosome aberrations (Bartsch *et al*., 1982; Nakamauro and Sayato, 1981; Siou *et al*., 1981; Barrett, 1982; IARC Monographs, Supplement 4, October, 1982).

The results of these comparisons show that a battery of 3 or 4 short-term tests for genotoxic activity can detect the range of human susceptibility to chemical carcinogens whereas studies in neither mice nor rats (nor both combined) can correlate with the human susceptibility range.

TABLE 12. PREDICTIVE VALUE OF SHORT-TERM GENOTOXIC TESTS FOR MAMMALIAN GERM CELL MUTAGENS

Test	Agreement Between Qualitative Effects in Short-Term Tests and Germ Cell Mutagen Tests (Percent)	
Cytogenetics *in vitro*	88	(28/32)[a]
Spot Test in Mice	83	(15/18)
SCE	83	(20/24)
Rodent Micronucleus	82	(28/24)
Cytogenetics *in vivo*	79	(27/34)
Drosophila SLRL	76	(31/41)
Spermhead Abnormality	73	(19/26)
Mammalian cell *in vitro* mutation	72	(22/32)
E. coli WP$_2$	65	(15/23)
Ames Test	64	(28/44)
Yeast Mitotic Recombination	61	(22/36)
Bacterial DNA Repair Test	60	(21/35)

[a] (Number of correct responses/number of comparisons).

The interpretations possible from this table are limited because of those problems identified in Table 6. But even with these limitations, one can clearly see that the bioassays used by EPA or NAS to make cancer risk estimates would not be the most accurate in predicting germ cell mutation risk.

Source: ICPEMC Committee 1 Final Report, 1983.

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PART II

SECTION 1

SAMPLING TECHNIQUES FOR COMBUSTION EMISSIONS

DIRECT SAMPLING OF RAW EXHAUSTS

The cumbersome sampling equipment of the mid 1950's to early 1970's necessitated voluminous extraction and clean-up procedures. At that time, large sample amounts were required for the analytical procedures used and biological tests were performed with skin painting on whole animals.

In 1954, the extracts of gasoline engine particulate emissions recovered by filtering at the actual exhaust temperature were shown to cause cancer in mice by Kotin et al. Later, in 1957, Mittler and Nicholson enriched some of the gaseous PAH by condensation of exhausts from both gasoline and diesel engines, however, no particulate emission was recovered. Stenburg et al. in 1961 used condensation before filtering and applied this technique to different suspected PAH emission sources including the automobile. The principle of this method was employed by Begeman and Colucci in 1962 to collect both gaseous PAH and particle-associated PAH by placing a large water condenser before the filter package. This trapping system was dimensioned to enrich the total exhaust volume from a 364 cubic inch V-8 engine operated on a chassis dynamometer. Gross (1972) using similar sampling system investigated the impact of parameters such as engine deposits, PAH content of the fuel, and emission control devices on the emissions (Table 13). For these sampling systems, very little or no information is available concerning optimization arrangements or recovery data. Also, these earlier studies did not take into consideration the possibility of losses of volatile species from filter, artifact formation or degradation during sampling.

In 1972, Grimmer et al. chose a vertical glass condenser with a cooling area of 0.25 m^2 for collection of the total exhaust volume. They reasoned that the washing procedure would be simple, using the glass device, and that catalytic effects on the condensed material should be lower. Evaluation of this method indicates that the entrance temperature on the filter should not exceed 35°C if quantitative trapping of three-ring PAHs was intended. Prolonged sampling involves losses of certain species, caused either by volatilization or degradation in this method. The Grimmer method has been used extensively in The Federal Republic of Germany by car manufacturers. An improved vertical glass condenser was described in 1981 by Kraft and Lies for trapping total exhaust volume for both gasoline and diesel vehicles.

TABLE 13. POLYNUCLEAR AROMATIC HYDROCARBON (PAH) EMISSIONS[a]

	PAH Emission µg/km[b]					
	Vehicle A[c]		Vehicle B[c]		Vehicle C[c]	
	Low PAH Fuel[d]	High PAH Fuel[e]	Low PAH Fuel[d]	High PAH Fuel[e]	Low PAH Fuel[d]	High PAH Fuel[e]
BaP	2.4	5.8	0.7	1.3	0.4	1.0
BaA	4.4	9.0	1.5	2.8	1.0	2.5

[a] Data of Gross (1972); condensation before filter.

[b] Gross' data originate from different fuel types and deposit levels in the engine and were originally reported as µg/gallon fuel used. Values above represent mean values from several fuels, and the recalculations in µg/km were made by the authors on an estimated mileage of 21 miles/gallon from a 7 mode cycle.

[c] A = vehicle without emission control, 1966
B = vehicle with engine modification, 1968
C = vehicle with engine modification, 1978.

[d] Low PAH fuel: BaP $\leq$ 0.1 ppm

[e] High PAH fuel: BaP = 3 ppm

PROPORTIONAL SAMPLING

A proportional sampling system was used by Chipman and Massey in 1960 for sampling such gaseous pollutants as HC, CO and NOx. With development of more sensitive analytical methods and biological tests *in vitro*, this technique has become an interesting alternative to large sampling equipment. Most proportional sampling systems use a reference signal which reflects the amount of emitted exhaust. Stenberg *et al*. (1981), used a proportional system connected to a glass condenser followed by a glass fiber filter. The resulting filter temperature was approximately 50°C; the total exhaust volume that was sampled was typically 10%. Using this sampling equipment, Stenberg (1979) investigated the emissions from 34 vehicles, representing the three most popular makes in Sweden. Fifteen different PAH's were identified from both the particulate and gas phase. The emission of five selected PAH's is summarized in Table 14.

TABLE 14. EMISSION OBTAINED FROM FTP, COLD START AND HOT START[a]

	Emissions, μg/km					
	Vehicle A[b]		Vehicle B[b]		Vehicle C[b]	
	Cold	Hot	Cold	Hot	Cold	Hot
CpcdP	51	10	29	11	2	0.5
BaA	25	15	9	8	8	3
BaP	38	7	7	2	4	2
BghiP	44	8	13	6	13	5
Coronene	21	2	16	4	6	3

[a] Data of Stenberg (1979); condensation before filter.

[b] A = mean value of 12 vehicles
B = mean value of 13 vehicles
C = mean value of 12 vehicles

DILUTION TUNNEL SAMPLING

The sampling methods described above utilize raw gases for the determination of PAH, i.e., the emission has not been mixed with ambient air where possible reactions with the atmosphere might occur. When particles and gaseous exhaust enter the ambient air, as from automobiles and trucks, the mixture is quickly cooled and diluted. During this process, the overall temperature is reduced to the point that carbon particles begin to absorb condensing organic material. Still-further dilution may reduce the gas-phase hydrocarbon concentrations to the point that further absorption ceases to occur. A technique has been developed in order to simulate reactions with ambient air, i.e., a dilution tunnel. Dilution tunnel conditions may not achieve normal environmental conditions, however, because the rate of dilution is considerably less in the tunnel than under ambient conditions. A number of investigators have used air dilution tunnel techniques to achieve this simulation and a wide variety of systems has been shown to be reasonably effective in at least some sampling applications. Originally, the construction of such equipment was devoted to the measurement of lead emissions from gasoline vehicles (Habbi, 1973 and Newhall et al., 1981). However, the utility of the dilution tunnel has been extended and it is now used in almost all measurements of automotive emissions. Schuetzle (1983) and Huisingh et al. (1978) have described schematically the research dilution tube system used in their laboratories (Figures 4 and 5). The dilution tube system described by Schuetzle (1983) includes an exhaust inlet manifold and mixing baffle, a dilution tube, and various down-stream probes and equipment. The dilution tube receives the exhaust as input, and supplies a known and reproducible quantity of filtered and humidity- and temperature-controlled ambient air for rapidly

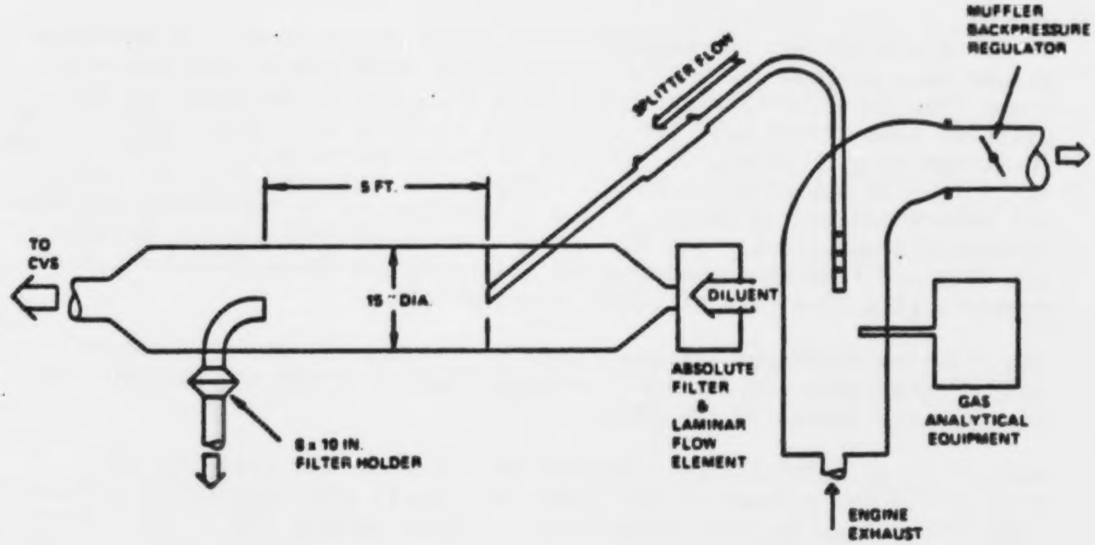

MUFFLER BACKPRESSURE REGULATOR
SPLITTER FLOW
5 FT.
TO CVS
15" DIA.
DILUENT
ABSOLUTE FILTER & LAMINAR FLOW ELEMENT
GAS ANALYTICAL EQUIPMENT
8 x 10 IN. FILTER HOLDER
ENGINE EXHAUST

Figure 4. Dilution tunnel system for collection of heavy duty diesel particulate (Husingh et al., 1978).

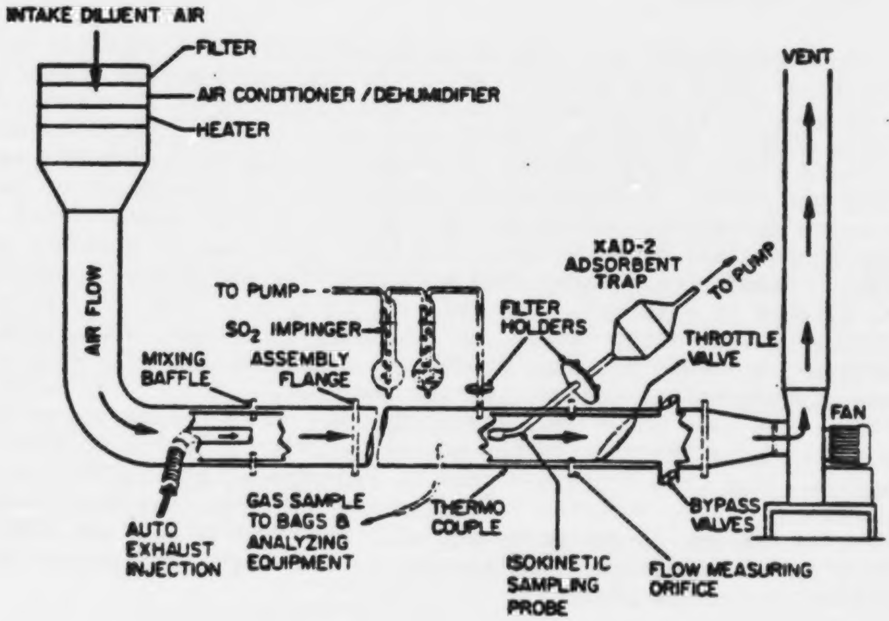

INTAKE DILUENT AIR
FILTER
AIR CONDITIONER / DEHUMIDIFIER
HEATER
VENT
XAD-2 ADSORBENT TRAP
TO PUMP
TO PUMP
SO_2 IMPINGER
FILTER HOLDERS
THROTTLE VALVE
AIR FLOW
MIXING BAFFLE
ASSEMBLY FLANGE
FAN
AUTO EXHAUST INJECTION
GAS SAMPLE TO BAGS & ANALYZING EQUIPMENT
THERMO COUPLE
BYPASS VALVES
ISOKINETIC SAMPLING PROBE
FLOW MEASURING ORIFICE

Figure 5. Dilution tube used for the collection of gas and particulate-phase emissions (Schutzle, 1983).

diluting and cooling the exhaust. The mixed stream flows at a measured volume rate through the dilution tube where fixed proportions are withdrawn from the tube isokinetically into the sampling devices. In the dilution tube system used for collection of heavy-duty diesel particulate (Huisingh et al., 1978), the exhaust is first variably split, then diluted 10-15 fold in the dilution tunnel. The individual mode dilution ratios are determined by the ratios of gas concentrations in the dilute and raw exhaust streams for CO_2 and NO. Large samples of particulate material are obtained from this apparatus by filtering the whole dilution tunnel contents at a flow rate of 12000 liters/minute.

The dilution technique has been used for a number of years. However, only limited data are available on measurements of PAH and related components (see Tables 15 and 16).

Newhall et al. (1981) have used another technique for sampling PAH. Dilution of the exhaust is performed in a small tube approximately 0.1x5 m long rather than in a dilution tunnel. Approximately 10% of the exhaust/air mixture was taken through two condensers in series, followed by a scrubbing tower and reaching finally an absolute filter. They have shown that the recovery of radioactive B(a)P added to the exhaust was better than 90% by this method. They also emphasize that the degradation of the exhaust would be minimum by trapping at subambient temperatures.

GAS PHASE TRAPPING

Gas phase components not trapped by Teflon membrane filter (or other filters) at 125°F are trapped, either by absorbant method (Spindt, 1974; Lee et al., 1979) or by a cryo-technique (Stenberg et al., 1981; Pedersen et al., 1980). Lee et al. (1979) used XAD-2 traps on gasoline emissions and found that a large portion of three-ring and four-ring PAH were present in the gas phase. A cryogradient system for the enrichment of gaseous PAH's and derivatives of these, in combination with conventional particle trapping on glass fiber filters, has been described in Stenberg et al. (1983). Samples were taken from both gasoline and diesel exhausts, which were diluted in a tunnel. The sampling equipment (Figure 6) consists of a thermostatted filter holder, an ice/water condenser, a CO_2/ethanol condenser, a liquid nitrogen condenser, a pump system with flow elements, temperature/pressure sensors, valves and dry-gas meter. By using this system, they have shown that compounds like phenanthrene and pyrene were present at more than 50% in the gas phase after dilution of gasoline exhausts. The cryo-technique used by Pedersen et al. (1980), consisted of two washing flasks immersed in liquid nitrogen and they have shown that five-ring PAH and larger molecules were absorbed to 100% on particles from gasoline emissions. Anthracene, fluoranthene, and pyrene were predominant in the gas phase.

TABLE 15. EMISSION OBTAINED FROM FTP[a,b]

	Emissions, µg/km					
	Diesel		Gasoline		Gasoline, Catalyst	
	A[c]	B[d]	A[c]	B[d]	A[c]	B[d]
Pyrene	15	ND[e]	8	ND[e]	0.7	ND[e]
BaP	0.5	3.9	3	3.2	0.1	<0.1
6 NO_2-B(a)P	<0.1	ND[e]	0.9	ND[e]	<0.1	ND[e]
1 NO_2-Pyrene	2	ND[e]	0.1	ND[e]	<0.1	ND[e]

[a] Data of Williams and Swarin (1981) and Gibson (1982).

[b] Only particulate emission from dilution tunnel.

[c] A = Gibson, one vehicle, hot start.

[d] B = Williams and Swarin. Diesel: mean value of two vehicles; gasoline: mean value of seven vehicles; gasoline catalyst: mean value of four vehicles, cold start.

[e] ND = not determined.

TABLE 16. EMISSIONS FROM FTP, COLD START[a,b]

	Emissions, µg/km			
	Gasoline	Gasoline, Catalyst	Gasoline M15[c]	Diesel
Phenanthrene	1.7	0.06	2.3	223
Fluoranthene	12.0	0.07	3.3	210
Pyrene	20.0	0.2	6.3	263
BghiF[d]	6.9	0.07	4.7	48
CPcdP	4.5	0.06	3.2	3.4
BaA	6.2	0.06	2.0	34
Chrysene	6.9	0.2	2.5	46
BbkF[e]	6.8	0.4	3.0	37
BeP[f]	5.5	0.3	3.2	33
BaP	1.7	0.1	1.1	11
InsP[g]	1.3	0.3	1.7	14
BghiP	3.9	0.7	5.2	22
Coronene	1.7	0.8	4.4	12

[a] Data of Egeback (1982).

[b] Only particulate sample from dilution tunnel. Mean value from three tests; diesel two tests.

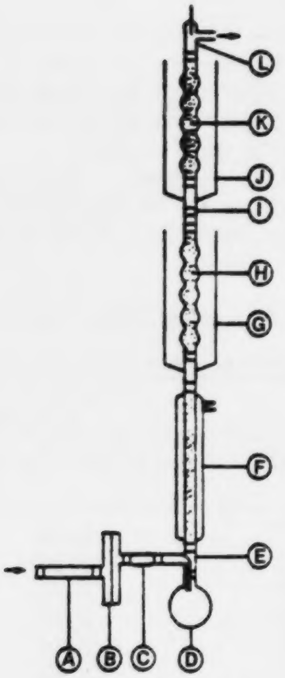
[c] Gasoline blended with 15% methanol.

[d] BghiF = Benzo(ghi)fluoranthene.

[e] BbkF = Benzo(b and k)fluoranthene.

[f] BeP value includes also benzo(cd)pyrenone.

[g] Ind P = Indeno (1,2,3-cd)pyrene.



(A) flexible metal tubing. 250 mm, (B) filter holder, diameter 127 mm, stainless steel; (C) Teflon bellows; (D) round-bottomed flask, 1000 mL; (E) normal glass joint; (F) ice/water condenser; (G) cryo vessel, dry ice/ethanol; (H) condenser cartridge; (I) ball glass joint; (J) cryo vessel, liquid nitrogen; (K) condenser cartridge; (L) Teflon/glass joint, temperature sensor.

Figure 6. Cryogradient sampling equipment, Stenberg et al., 1983.

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PART II

SECTION 2

EXTRACTION METHODS FROM EMISSION PARTICULATES

Diesel particulate emissions are a very complex mixture of carbonaceous matter containing adsorbed and/or condensed organic components from the combustion of fuel and lubricants. The organic solvent extractable fraction of these particulates is extremely complex and has been reported to contain hundreds of individual components. A typical experimental approach used in the characterization of the soluble organic fraction of diesel particulates involves extraction of the organic matter from the collected particulate using an organic solvent, concentration of the extract for subsequent analysis, fractionation into less complex non-polar and polar subgroups and, final concentration to facilitate chemical and biological analysis.

EXTRACTION

Two widely employed methods for removal of organics associated with diesel particulates are Soxhlet extraction and ultrasonic extraction using a suitable organic solvent. Petersen *et al*. (1980) have indicated that either technique can be used satisfactorily to measure the total soluble organic fraction. However, the total quantity of material extracted by these two methods is influenced by the type of particulate being extracted, contact time with the solvent, temperature of the solvent during extraction, and polarity of the solvent. Extraction by sonication is usually more rapid (about 2 hours) than using the Soxhlet technique (3-24 hours) but sonication requires several additional analytical steps to remove suspended soot and filter particles. Soxhlet extraction gives slightly higher mass and B(a)P recovery than ultrasonication extraction (Schuetzle and Perez, 1981). Soxhlet extraction can be conducted unattended and many particulate samples can be extracted simultaneously in separate apparatuses.

The type of organic solvent used for extraction has received much attention. Solvents like methanol and aromatic-alcohol mixtures have been found to extract the largest quantities of material from the particulate, presumably because of increased extraction of inorganic material (Siak *et al*., 1979; Brooks *et al*., 1979; Swarin and Williams, 1979). Binary solvent systems using an aromatic solvent and alcohol give the highest recovery of mass and B(a)P when compared to most other solvent systems (Schuetzle, 1983). Aromatic solvents have been shown to extract the largest quantity of PAH (Choudhury and Doudney, 1979). The largest quantity of biologically active material from particulates is extracted by dichloromethane and is usually the solvent chosen for biological screening (Huisingh et al., 1978; Brooks et al., 1979).

CONCENTRATION

Concentration is necessary within various steps of the overall procedure. The solvent must be removed from the extract, or an aliquot of the extract, to determine the residue mass for calculating the total organic mass. Typical methods of concentration include removal of the solvent by rotary film evaporation (Petersen et al., 1980), nitrogen stream blow-down (Swarin and Williams, 1979; Petersen et al., 1980) and Kuderna-Danish concentration. For removing large quantities of solvent (1000 ml to 10 ml) rotary film evaporation is the most widely used method. Nitrogen stream evaporation is used to concentrate the solvent to several hundred microliters or to dryness. Kuderna-Danish concentration has not received much attention for the concentration of particulate extracts primarily because of the overall time of concentration. Another method of concentrating small volumes (10 ml to 1 ml or less) is by vortex evaporation. Using this method, the solvent is transferred to a centrifuge tube, which is placed in the vortex unit. During the vortex mixing, the solvent is removed while heating (20-60°C) under vacuum.

FRACTIONATION

Before chemical or biological characterization, fractionation of the particulate extract may be performed. For fractionation of the particulate extracts on the basis of chemical functionality, a variety of partitioning methods and chromatographic techniques can be used (Huisingh et al., 1978; Swarin and Williams, 1979; Choudhury and Dondney, 1979; Lyons, 1962).

By sequential liquid-liquid partitioning, acids and bases can be removed from the neutrals using aqueous solutions at low and high pH. Neutral organic compounds are further fractionated by solvent partitioning and/or column chromatography. Column techniques using Sephadex LH-20, silica gel and alumina have been developed to separate the neutrals based on compound classes. For separation of acids, bases, neutral nitrogen-containing compounds, saturated hydrocarbons and aromatic hydrocarbons, Jewell et al. (1972) have used a combination of ion-exchange coordination and adsorption chromatography. Rapid fractionation of particulate extracts by the use of high performance liquid chromatography (HPLC) has been reported by Huisingh et al. (1978). This method was modified by Schuetzle and co-workers (1981) to produce six specific fractions.

BIOLOGICAL EXTRACTION

Organic solvents such as dichloromethane readily remove the organic compounds from the diesel exhaust particles; the extracted material is mutagenic to bacterial and mammalian cells. However, organic solvents used for extraction are not typical of the solvents present within biological systems such as the respiratory tract. Biological fluids have been used as solvents to extract the organic compounds, however, the removal process is not nearly so efficient as with dichloromethane. Siak *et al.* (1980) and Brooks *et al.* (1980) have demonstrated that, when fluids compatible with the internal environment of the human body have been used instead of the industrial organic solvents for extraction, mutagenic activity was significantly reduced. The mutagenic activity extracted from diesel particulates by typical body fluids such as blood serum or a solution of blood proteins is entirely negligible in comparison with that extracted by an organic solvent. King *et al.* (1981) confirmed that organic solvents are more efficient than physiological fluids in removing mutagens from diesel particles and reported also that the activity of hydrocarbons extracted with dichloromethane is greatly reduced upon addition of serum and lung cytosol. Subsequent incubation of serum and cytosol-bound organics with protease increased the mutagenic activity. One explanation for these observations is that the compounds responsible for the mutagenic activity were inactivated or firmly bound to the proteins and do not exert biological activity but could be released by the protease and become available to the test system. Later studies by King *et al.* (1983) showed that lung macrophages can apparently remove the mutagenic nitroaromatic materials from engulfed diesel exhaust particles.

Li *et al.* (1981) also reported that organic materials dissociate from particles much more slowly *in vivo* than when extracted by organic solvents *in vitro* and that serum and tissue cytosols significantly reduce the cytotoxicity of diesel particulate extracts. From 0-8% of mutagenic activity extracted by dichloromethane was obtained by incubation of particles with biologically relevant solutions like lavage fluids or serum. Quantitative studies of the dissociation of benzo(a)pyrene from particles indicated that, although 65% of the benzo(a)pyrene content was eluted by ethanol in 1 hour, none was eluted by saline, and only 12% was recovered after 24 hours perfusion of particles with 1:1 diluted serum. The authors concluded that biologically relevant solvents may bind or detoxify mutagenic compounds and make them unavailable for the test system.

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PART II

SECTION 3

REVIEW OF BIOASSAYS APPLIED TO UNREGULATED COMBUSTION EMISSION SAMPLES

INTRODUCTION

Most of the data describing the activity of automobile emissions in a broad spectrum of bioassays have been presented and analyzed in four publications.

1. The Report of the Health Effects Panel of the Diesel Imports Study Committee, National Research Council, Health Effects of Exposure to Diesel Exhaust, National Academy Press, Washington, D.C., 1981.

2. Toxicological Effects of Emissions from Diesel Engines, J. Lewtas, editor, Elsevier Biometical Press, New York, 1982. Proceedings of the EPA 1981 Diesel Emissions Symposium held in Raleigh, North Carolina, October 5-7, 1981.

3. Environmental Health Perspectives, volume 47, January, 1983. Mutagenicity and carcinogenicity of air pollutants. The proceedings of the Symposium on Biological Tests in the Evaluation of Mutagenicity and Carcinogenicity of Air Pollutants with Special Reference to Motor Exhausts and Coal Combustion Products, held in Stockholm, Sweden, February 8-12, 1982.

4. Environmental International, Volume 5, 1981. A series of articles summarizing several years of investigation.

The objective of this section is to summarize both the extent of testing and the qualitative responses for automobile emission products in short-term tests for mutagens and carcinogens. A concomitant objective is to attempt to select bioassays which are most suitable for routine analysis of emissions from new mobile sources based on the results of the tests.

Review of the bioassay data is organized by the type of toxic endpoint measured and is subdivided into mammalian in vivo tests and in vitro mammalian/submammalian tests. Table 17 outlines the stratification of endpoints included in this evaluation.

Evaluation of data from the bioassay data base will be examined using the procedure outlined in Figure 7. The end product of this procedure will be a battery of tests amenable to routine evaluation of emissions.

Data relevant to the activity of specific fractions of particulate extracts and to extracts derived from various fuels and engine running conditions will not be addressed in this section because those parameters are reviewed in detail in other sections of the report.

Tables 18-28 lists the reported responses of emission samples from diesel and gasoline powered engines. Each matrix consists of either the positive or negative data elements for a genetic or carcinogen bioassay. The absence of an entry in a square indicates a gap in the data base.

OVERVIEW OF BIOASSAY RESPONSE

The summary of positive and negative results by endpoint given in Tables 18 through 28 includes the qualitative responses for all studies reported for automotive exhaust emissions.

In addition to the obvious data gaps, some apparent significant contradictions in qualitative responses exist in the total data set shown in the previous set of tables. These are as follows:

TABLE 17. STRATIFICATION OF BIOASSAY ENDPOINTS REVIEWED

Genotoxic Effects

A. Specific Locus Mutation in Prokaryotic and Eukaryotic Organisms

B. Chromosome Alterations (Including Aberrations, Sister Chromatid Exchange, and Aneuploidy)

C. Damage at the Primary DNA Level in Mammalian Cells *In Vivo* and *In Vitro* (Including DNA Adduct Formation, DNA Repair Phenomena, Mitotic Crossing Over)

Cell Transformation and/or Tumor Induction

A. *In Vitro* Cell Transformation in Mouse Cell Lines and Hamster Primary Cells

B. Short-Term Tumor Induction Assays in Mice

C. Long-Term Tumor Induction Assays in Rodent Species

D. Co-Carcinogenesis Studies in Rodents

STEP		ACTION
Collection, Collation and Review of Available Bioassay Data		Preparation of Citation Index
	↓	
Analysis of Each Data Element in a Response Matrix Organized by Endpoint		Extraction of Qualitative Response, Dose Range, Test Conditions, Sample Source and Quantitative Effects
	↓	
Summary of all Qualitative Reponses (Positive and Negative)		Comparison of sample type, sample source and quantitates response by assay. Tables 18 through 28 provide rapid analysis of data by end-point.
	↓	
Identify Bioassays Capable of Detecting Genotoxic/ Carcinogenic Effects of Automotive Emissions		Table 29 summarizes the responses from Tables 18 through 28.
	↓	
Critique of All Bioassays Producing Positive Responses		Tables 30 through 33 show detailed analysis and critique of positive studies by endpoint.
	↓	
Distribution of Positive Bioassay Responses Across Sample Types		Table 34 identifies tests most amenable to a broad range of sample types.
	↓	
Selection of Candidate Bioassays for use in Emission Screening		Tests which meet all previously described criteria of a good screening test and are responsive to the broadest range of sample types and sources will be considered candidate screening tests.

Figure 7. Bioassay data evaluation matrix.

TABLE 18. MATRIX OF POSITIVE RESPONSES FOR GENE MUTATION

	DIESEL		GASOLINE	
	LIGHT DUTY	HEAVY DUTY	LIGHT DUTY	HEAVY DUTY
WHOLE EXHAUST	· Tradescantia			
VAPOR PHASE ONLY				
UNEXTRACTED PARTICULATES	· CHO/HGPRT Mutation			
BIOLOGICALLY EXTRACTED PARTICULATES	· Ames Assay			
ORGANIC SOLVENT EXTRACTED PARTICULATES	· Mouse Lymphoma Assay · *E. coli* WP$_2$ uvrA mutation · Ames Forward and Reverse · Human Lymphoblast/HGPRT Mutation · CHO/HGPRT Mutation Assay · *E. coli* K12 Forward Mutation · Human XP cells HGPRT Mutation	· Mouse Lymphoma Assay · Ames Assay · CHO/HGPRT Mutation Assay	· Mouse Lymphoma Assay · Ames Assay · CHO/HGPRT Mutation Assay	· Ames Assay · Mouse Lymphoma Assay

HGPRT = Hypoxanthine guanine phosphorbosyl transferase
XP = Xeroderma pigmentosum
CHO = Chinese hamster ovary cell line

TABLE 19. MATRIX OF NEGATIVE RESPONSED FOR GENE MUTATION

	DIESEL		GASOLINE	
	LIGHT DUTY	HEAVY DUTY	LIGHT DUTY	HEAVY DUTY
WHOLE EXHAUST	· Mouse Specific Locus Assay · Drosophila SLRL Assay			
VAPOR PHASE ONLY	· Drosophila SLRL Assay			
UNEXTRACTED PARTICULATES	· Ames Assay			
BIOLOGICALLY EXTRACTED PARTICULATES	· Ames Assay			
ORGANIC SOLVENT EXTRACTED PARTICULATES	· V79/HGPRT Mutation Assay · Drosophila SLRL Assay · Yeast Reverse Mutation in Strain D7	· CHO/HGPRT Mutation Assay		· Mouse Lymphoma Assay

SLRL = Sex linked recessive lethal
V79 = Chinese hamster lung cell line

TABLE 20. MATRIX OF POSITIVE RESPONSES FOR CYTOGENETICS ASSAYS

	DIESEL		GASOLINE	
	LIGHT DUTY	HEAVY DUTY	LIGHT DUTY	HEAVY DUTY
WHOLE EXHAUST	· Tradescantia Micronucleus Assay · SCE in Hamster Lung Cells			
VAPOR PHASE ONLY				
UNEXTRACTED PARTICULATES	· SCE in Mouse Bone Marrow Cells · SCE in Hamster Lung Cells			
BIOLOGICALLY EXTRACTED PARTICULATES				
ORGANIC SOLVENT EXTRACTED PARTICULATES	· SCE in Mouse Bone Marrow Cells · SCE in Fetal Liver Cells · SCE in Hamster Lung Cells · Aberrations in Lymphocytes	· SCE in CHO Cells · Aberrations in vitro in CHO Cells	· SCE in CHO Cells · Aberrations in CHO Cells	· SCE in CHO Cells

SCE = Sister chromatid exchange

TABLE 21. MATRIX OF NEGATIVE RESPONSES FOR CYTOGENETICS ASSAYS

	DIESEL		GASOLINE	
	LIGHT DUTY	HEAVY DUTY	LIGHT DUTY	HEAVY DUTY
WHOLE EXHAUST	· Mouse Micronucleus Assay · Hamster Micronucleus Assay · SCE in Mouse Bone Marrow Cells · SCE in Fetal Liver Cells · Mouse Dominant Lethal Assay · Mouse Heritable Translocation Assay · SCE in Dog Lymphocytes · Micronucleus in Dog Lymphocytes			
VAPOR PHASE ONLY				
UNEXTRACTED PARTICULATES	· Mouse Micronucleus Assay · Hamster Micronucleus Assay · SCE in Fetal Liver Cells			
BIOLOGICALLY EXTRACTED PARTICULATES				
ORGANIC SOLVENT EXTRACTED PARTICULATES	· Hamster Micronucleus Assay · Mouse Micronucleus Assay			

TABLE 22. MATRIX OF POSITIVE RESPONSES FOR PRIMARY DNA DAMAGE

	DIESEL		GASOLINE	
	LIGHT DUTY	HEAVY DUTY	LIGHT DUTY	HEAVY DUTY
WHOLE EXHAUST				
VAPOR PHASE ONLY				
UNEXTRACTED PARTICULATES	· DNA Repair in Human XP Cells			
BIOLOGICALLY EXTRACTED PARTICULATES				
ORGANIC SOLVENT EXTRACTED PARTICULATES	· DNA Repair in XP Human Fibroblast Cells · UDS in EUE Human Cells · UDS in Rat Primary Hepatocytes · UDS in tracheal epithelium of hamsters · Yeast (D7) Mitotic Recombination	· UDS in Rat Primary Hepatocytes	· DNA Breakage in SHE Cells	

XP = Xeroderma pigmentosum
UDS = Unscheduled DNA Synthesis
SHE = Syrian hamster embryo

TABLE 23. MATRIX OF NEGATIVE RESPONSES FOR PRIMARY DNA DAMAGE

	DIESEL		GASOLINE	
	LIGHT DUTY	HEAVY DUTY	LIGHT DUTY	HEAVY DUTY
WHOLE EXHAUST				
VAPOR PHASE ONLY				
UNEXTRACTED PARTICULATES	· DNA Repair in Human XP Cells			
BIOLOGICALLY EXTRACTED PARTICULATES				
ORGANIC SOLVENT EXTRACTED PARTICULATES	· Yeast Mitotic Recombination in Strain D3 · DNA Breakage in SHE Cells	· DNA Breakage in SHE Cells · UDS IN WI-38 Human Cells		

XP = Xeroderma pigmentosum
SHE = Syrian hamster embryo

TABLE 24. MATRIX OF POSITIVE RESPONSES FOR IN VITRO TRANSFORMATION ASSAYS

	DIESEL		GASOLINE	
	LIGHT DUTY	HEAVY DUTY	LIGHT DUTY	HEAVY DUTY
WHOLE EXHAUST				
VAPOR PHASE ONLY				
UNEXTRACTED PARTICULATES				
BIOLOGICALLY EXTRACTED PARTICULATES				
ORGANIC SOLVENT EXTRACTED PARTICULATES	· Enhancement of Viral Transformation in SHE Cells · Balb/c 3T3* Cell Transformation		· Enhancement of Viral Transformation in SHE Cells · Balb/c 3T3 Cell Transformation*	

*Equivocal response
SHE = Syrian hamster embryo

TABLE 25. MATRIX OF NEGATIVE RESPONSES FOR IN VITRO TRANSFORMATION ASSAYS

	DIESEL		GASOLINE	
	LIGHT DUTY	HEAVY DUTY	LIGHT DUTY	HEAVY DUTY
WHOLE EXHAUST				
VAPOR PHASE ONLY				
UNEXTRACTED PARTICULATES				
BIOLOGICALLY EXTRACTED PARTICULATES				
ORGANIC SOLVENT EXTRACTED PARTICULATES	· Balb/c 3T3 Cell Transformation	· Enhancement of Viral Transmation in SHE Cells	· Balb/c 3T3 Cell Transformation	· Balb/c 3T3 Cell Transformation

SHE = Syrian hamster embryo

TABLE 26. MATRIX OF POSITIVE RESPONSES FOR *IN VIVO* TUMOR STUDIES

	DIESEL		GASOLINE	
	LIGHT DUTY	HEAVY DUTY	LIGHT DUTY	HEAVY DUTY
WHOLE EXHAUST	· Respriatory Co-Carcinogenesis Studies with DEN in hamsters			
VAPOR PHASE ONLY				
UNEXTRACTED PARTICULATES				
BIOLOGICALLY EXTRACTED PARTICULATES				
ORGANIC SOLVENT EXTRACTED PARTICULATES	· Subcutaneous injection site tumors in mice · Skin tumor initiation in Sencar mice		· Skin tumor initiation in Sencar mice	

DEN = Diethylnitrosamine

TABLE 27. MATRIX OF NEGATIVE RESPONSES FOR *IN VIVO* TUMOR STUDIES

	DIESEL		GASOLINE	
	LIGHT DUTY	HEAVY DUTY	LIGHT DUTY	HEAVY DUTY
WHOLE EXHAUST	· Liver focus assay in mice · Hamster respiratory tract tumors · Lung tumors in Strain A mice · Lung tumors in Sencar mice			
VAPOR PHASE ONLY	· Hamster respiratory tract tumors			
UNEXTRACTED PARTICULATES	· Skin tumor initiation in C3H mice			
BIOLOGICALLY EXTRACTED PARTICULATES				
ORGANIC SOLVENT EXTRACTED PARTICULATES	· Skin tumor initiation in Sencar mice · Skin tumor initiation in C3H mice · Complete Carcinogen in Sencar mice			

TABLE 28. LONG-TERM STUDIES OF THE HEALTH EFFECTS OF DIESEL EXHAUST, COMPLETED OR IN PROGRESS

Laboratory	Species	Particle Concentration (μg/m^3)	Response
Environmental Protection Agency	Chinese Hamster, Mice, Rats, Cats	6000-12000	Negative
Fraunhofer Institute	Syrian Hamsters	4200[a]	Negative
General Motors	Rats, Guinea Pigs	250, 750, 1500	Negative
Lovelace Inhalation Toxicology Research Institute	Mice, Rats	350, 3500, 7000	Negative
Battelle-Northwest	Rats Syrian Hamsters	8300 7300	Negative
Southwest Research Institute	Syrian Hamsters, Rats, Mice	1:60, 1:120, 1:360 dilution[b]	Negative

[a] Also, exposures to gaseous emissions only without particles.
[b] Particle concentrations not given.

From McClellan *et al.*, 1982.

GENE MUTATION BIOASSAYS

Organic solvent extracts of light duty diesel particles were mutagenic in CHO cells but not V-79 cells. Both cell lines are derived from Chinese hamsters and generally respond similarly. Attempts should be made to resolve this apparent discrepancy, especially since there was also a contradictory response in CHO cells with heavy duty diesel solvent extracts.

CYTOGENETIC BIOASSAYS

One series of studies in which SCE was measured in bone marrow cells indicated a much stronger response for whole exhaust particles injected intraperitoneally in DMSO than for organic solvent extracts of the particles injected intraperitoneally. Considering that significant differences in bioavailability of mutagens was anticipated between these two samples, the responses reported are surprising because they contradict the anticipated responses. The test procedures and data from these two studies should be reviewed in detail.

PRIMARY DNA EFFECTS

Most of the responses reported were consistent throughout all bioassays. The only apparently inconsistent effects were reports of mitotic recombination. Studies using yeast strain D3 were reported as positive for organic solvent extracts of diesel emission particles whereas results for extracts using yeast D7 were reported to be weakly positive. The source of diesel emissions was different in the studies and may account for this apparent discrepancy. A comparison of both sets of data should be made in an attempt to resolve the question.

CARCINOGENESIS STUDIES

There appeared to be considerable variability among the responses for direct transformation studies in Balb/c 3T3 cells. The ability of organic solvent extracts to produce transformation varied considerably, which possibly indicated an engine type specificity. The variable results might also indicate test system variability, especially since the positive responses reported were neither generally dose dependent nor strong. A systematic analysis of this body of data should be made using a properly selected statistical approach.

Among the in vivo studies it was established that organic solvent extracts from light duty diesel emissions could initiate skin tumors in SENCAR mice but not in C3H mice. Both mice were susceptible to PAH control agents, but the SENCAR mice were selected because they were sensitive to early and repoducible apperance of papillomas after PAH initiation and TPA protomotion.

ANALYSIS BY TYPE OF SAMPLE TESTED

The data base for whole exhaust, vapor phase, or unextracted particles is extremely limited and drawing generalizations from the data base will be extremely restrictive, especially since most of the findings are negative. Table 29 describes the distribution of positive responses according to parameters of bioassay endpoint, sample type, and engine type. It is clear that most of the bioassay data consist of studies with light duty diesel particulates extracted with organic solvents.

EVALUATION OF POSITIVE RESPONSES

In order to use the current bioassay experience, for the purpose of selecting useful tests of automobile emissions in a certification program, the results from those bioassays able to detect biological activity must be examined in detail. Tables 30 through 33 provide an analysis of the results from bioassays using one of the emission types listed in Table 29. The results are organized by endpoint, relevant references, test conditions employed, and test response; specific comments are made when appropriate.

CRITERIA FOR BIOASSAY SUITABILITY FOR EVALUATING MOTOR VEHICLE EMISSION SAMPLES

In making an assessment of bioassay suitability, several parameters were considered.

These are as follows:

1. Test Sample Compatibility - demonstrated adaptability to samples other than organic solvent extracts is considered a desirable feature.

2. Relevance of endpoint - this parameter is not critical in a screening mode but would become more important if the results from bioassays are extrapolated to specific toxic endpoints. Tests directly assessing tumorigenicity, for example, would be preferable in estimating the potential carcinogenic hazard of a sample than a test for unscheduled DNA synthesis (UDS).

3. Relevance of exposure route - this parameter assumes significance in assessments that require extrapolation to ambient exposures and risk estimates.

4. Relevance of the target cell - mammalian cells might well be expected to provide greater reliability for extrapolation purposes than would bacteria, yeast, insect or plant cells. While these latter cell types offer distinct advantages in some instances, some bioassays using non-mammalian cells may be inappropriate for evaluating combustion emissions.

5. Reproduciblity and quantitation - assays which do not provide reproducible responses under identical treatment conditions with one sample would not be useful in routine screening of new emission sources where variables such as engine and fuel types may produce subtle and important variations in response patterns. Quantitative data analysis and induction of dose-dependent responses are also important criteria.

TABLE 29. DISTRIBUTION OF BIOLOGICAL ENDPOINTS EXAMINED ACROSS AUTOMOTIVE ENGINE TYPES

Endpoint	Diesel Engine						Gasoline Engine[a]	
	Light Duty					Heavy Duty	Light Duty	Heavy Duty
	WE	VP	UP	OP	BP	OP	OP	OP
A. Gene Mutation	X	X	X[b]	X	X	X	X	X
B. Chromosome	X		X	X		X	X	X
C. Primary DNA Damage			X	X		X	X	
D. Carcinogenicity Studies								
1. _In_ _vitro_ Transformation				X		X	X	
2. _In_ _vivo_ Initiation	X	X	X	X		X		
3. _In_ _vivo_ Co-carcinogen	X							
4. _In_ _vivo_ Promotion				X			X	

WE = whole exhaust
VP = vapor phase only
UP = unextracted particulate phase only
BP = biologically extracted particulate phase
OP = organic solvent extracted particulate phase

[a] Most of the data employed came from engines using leaded gasoline.
[b] When tested in cell capable of engulfing the particles.

TABLE 30. MOTOR VEHICLE EMISSIONS: A SUMMARY OF POSITIVE GENE MUTATION BIOASSAY RESULTS

Bioassay	Reference	Test Conditions	Response	Comments
Specific locus mutation in coliform bacteria	Claxton, 1982 Liber *et al*, 1981 Claxton, 1981 Lewtas, 1983 Wang *et al*., 1981	Forward and reverse mutation tests have been reported for *Salmonella typhimurium*, and for reverse mutation in *E. coli* WP_2 uvrA. Nonactivation and activation test conditions using organic solvent and biological fluid extracts of emission particles have been run. Both LD and HD diesel and gasoline engines run on a range of different fuels were used. Recent studies have been conducted with vapor phase components.	The *Salmonella* assays indicate the presence of potent direct acting bacterial mutagens in organic solvent extracts of all types of emission particles. The response range is broad and depends to great extent on variables such as engine, fuel and collection conditions. Recent studies have also demonstrated the presence of bacterial mutagens in the vapor phase of automotive emission.	While the addition of S9 tended to reduce the biologial activity of diesel samples in the *Salmonella* tests, it virtually eliminated activity in the *E. coli* bioassay. Gasoline samples show enhanced mutagenicity in the presence of S9.
Specific locus mutation in cultured mammalian cells				
1. Mouse Lymphoma (TK) L5178Y	Lewtas, 1983 Mitchell *et al*, 1981 U.S. EPA, 1983	This bioassay is a forward mutation assay using an autosomal gene (TK). The test is conducted under nonactivation and activation test conditions. Tests were reported for organic solvent extracts of LD and HD diesel and gasoline engine emissions.	This assay responds to the mutagens present in organic extracts of all engine types. Dose related increases are typically generated from both activation and nonactivation test conditions.	Diesel samples generally do not require S9 for maximum mutagenicity. Gasoline samples generally showed enhanced mutagenicity in the presence of S9.
2. Chinese Hamster Ovary (CHO) Cells	Li *et al*, 1982 Casto *et al*, 1981 Cheschier, *et al*. 1980	This bioassay uses an X-linked gene (HGPRT) to assess mutagenicity. Tests on LD diesel and gasoline engine emissions (organic solvent extracted) were conducted under nonactive test conditions.	CHO protocol modified by Li gave rise to positive and generally dose-dependent responses for both engine types. Mutagenic responses were observed over a 20-50 μg/ml dose range of the extract.	Earlier studies using the exposure protocol of HSIE, resulted in very weak to negative responses in the CHO test system with particle extracts. Whole particles were shown to be even more mutagenic after CHO cell engulfment.

TABLE 30. MOTOR VEHICLE EMISSIONS: A SUMMARY OF POSITIVE GENE MUTATION BIOASSAY RESULTS
(Continued)

Bioassay	Reference	Test Conditions	Response	Comments
3. Human lymphoblast cells	Liber et al, 1981 Barfnecht et al, 1982	This bioassay employs the HGPRT locus described above. Studies with and without metabolic activation were conducted using organic solvent extracted LD diesel particles from one vehicle.	The results were positive, but only under activation test conditions. The active dose range was 10-20 μg/ml of the extract.	This is the only report of a mammalian cell assay exhibiting an S9-dependent response for diesel emissions extract.
4. Human XP fibroblast cells	McCormick et al, 1980	This bioassay employs the HGPRT locus described above; however, the fibroblast cells employed were explants from an individual with xeroderma pigmentosum and are deficient in DNA repair. Only nonactivation test conditions were employed. The samples tested were unextracted and organic solvent extracted LD diesel particles.	The tests for both particles and extracts were reported positive in the absence of metabolic activation.	This is a report of unextracted particles inducing mutation in cultured mammalian cells.
5. Balb/C 3T3 fibroblast	Curren et al, 1981	Ouabain resistance (dominant mutation) using organic solvent extracts of diesel and gasoline engines. Tests included S9 and non-S9 studies.	Results were equivocal. No dose response results reported.	The report indicated possible positives for one diesel sample sample and one gasoline sample.
Tradescantia	Schairer et al, 1981	Tradescantia plants exposed to LD diesel whole exhaust emissions. Stamen hair cells are scored for specific locus mutation.	The assay was reported as positive.	

LD = Light duty
HD = Heavy duty

TABLE 31. MOTOR VEHICLE EMISSIONS: A SUMMARY OF POSITIVE PRIMARY DNA DAMAGE BIOASSAY RESULTS

Bioassay	Reference	Test Conditions	Response	Comments
DNA damage in human XP fibroblast explants	Barfnecht *et al*, 1982	LD diesel particles (unextracted) and organic solvent extracts from the particles were tested for direct DNA damage using a comparative cytotoxicity test.	The results for both samples were positive. No activation was required.	The primary reference for this study is McCormick *et al*., 1980, in Health Effects of Diesel Engine Emission, EPA symposium.
Unscheduled DNA Synthesis (UDS) in mammalian cells	National Academy Press, 1981 U.S. EPA, 1983	Organic solvent extracts from LD diesel emissions were tested in several mammalian cell lines and primary cells for their ability to stimulate the enzymatic DNA repair system. Tests in WI-38 and EUE cell lines were conducted with and without S9 mix. The primary cells used in one study were metabolically competent liver hepatocytes.	The results for the LD diesel samples were reported to be positive.	
Mitotic Recombination in Saccharomyces	U.S. EPA, 1983	Organic solvent extracts from a LD emission sample were tested in several yeast strains. The D7 strain combines three endpoints (mutation, mitotic gene conversion and mitotic recombination). Tests were all conducted under nonactivation and activation test conditions.	Sample was weakly positive under nonactivation test conditions in the mitotic recombination endpoint.	A similar study employing same endpoint in a different yeast strain (D3) was reported negative.
Unscheduled DNA Synthesis (UDS) in hamster tracheal organ culture	Schiff *et al*, 1981	The ability of an organic solvent extract from LD diesel emissions to elict UDS in primary tracheal cells located in an organ culture was analyzed using an autoradiographic technique.	The sample was positive at 1.0 and 10 µg/ml of the extract.	
DNA Breakage in Syrian Hamster Embryo (SHE) Cells	Casto *et al*, 1981	Organic solvent extracts of gasoline engine particulate tested *in vitro* in SHE cells. The endpoint was the sedimentation shift for DNA in alkaline sucrose density gradients.	Positive response at 250 ug/ml of gasoline particulate emission extracts.	Results from light duty diesel extracts negative.

TABLE 32. MOTOR VEHICLE EMISSIONS: A SUMMARY OF POSITIVE CYTOGENETIC BIOASSAY RESULTS

Bioassay	Reference	Test Conditions	Response	Comments
Micronucleus Assay in Tradescantia	Ma, et al 1981	Plants exposed to whole diesel exhaust (LD) and stamen hair cells scored for micronuclei.	Positive	
Sister chromatid exchange (SCE) in hamster lung cells	Guerrero and Rounds, 1981 Guerrero and Rounds, (in press)	SCE observed in explanted lung cells following exposure by inhalation and instillation to diesel emissions.	Positive at 12 mg/m^3 to whole diesel exhaust; particles were positive at 5.0 mg/animal, and organic extract of particles were positive at 0.6 mg/animal.	Statistical treatment of data showed significant p values.
Sister chromatid exchange (SCE) in mouse bone marrow cells	Pereira, 1982	Unextracted diesel particulates administered by I.P. at 300 mg/kg BW (DMSO was the solvent). Organic extracts of diesel particulates administered at 800 mg/kg BW.	The whole particles increased SCEs from 5.6/cell in the control to 11.3 at the active dose. The organic extracts increased the control value of 5.6 to 9.4/cell.	No statistical analyses were used to confirm these effects which were not found positive.
Sister chromatid exchange (SCE) in cultured mammalian cells	Mitchell et al, 1981 Lewtas, 1983 U.S. EPA, 1983	Tests conducted using organic solvents of diesel (LD and HD) and gasoline engines. Most tests were conducted both with and without S9 mix.	Organic solvent extracts of collected diesel and gasoline particulates are positive producing dose related increases. Metabolic activation was not required for SCE induction.	Positive effects were statistically significant. These data are supported by in vivo studies.
Chromosome aberrations in cultured mammalian cells	Lewtas, 1983 U.S. EPA, 1983	Tests were conducted without S9 in CHO cells using organic extracts of diesel and gasoline engines. Human lymphocytes were exposed to diesel organic extracts with and without S9 mix.	Chromosome aberrations were observed without S9 mix in both cell types. Active dose ranges were from about 1-10 μg/ml. The presence of S9 significantly reduced the clastogenic activity of diesel emission extracts.	These positive responses were not confirmed by in vivo studies scoring for chromosome aberrations.
Sister chromatid exchange (SCE) in fetal liver cells	Pereira, 1982	Pregnant Syrian hamsters were exposed to LD diesel whole exhaust, unextracted particles and solvent extracts of particles. Liver cells were removed from developing embryo and screened for SCE.	Organic solvent extracts were positive. The other two exposures did not produce effects. The exposures for the negative studies were 12 mg/m^3 x 12 days and 300 mg/kg BW x 1 day, respectively. No dose level(s) were given for the extract.	No data were shown for this study and its usefulness in evaluating the genotoxicity of diesel emissions must be seriously questioned.

BW = Body Weight

TABLE 33. MOTOR VEHICLE EMISSIONS: A SUMMARY OF POSITIVE CELL TRANSFORMATION BIOASSAY RESULTS

Bioassay	Reference	Test Conditions	Response	Comments
Viral enhancement of cell transformation (in vitro)	Casto, et al, 1981 Lewtas, 1983	Primary Syrian hamster embryo cell explants (containing SA7 virus) were exposed to organic solvent extracts of LD and HD diesel and gasoline engine emissions.	LD diesel emission extracts enhanced viral transformation in SHE cells over a dose range of 60-250 µg/ml. The HD sample was active at 500 µg/ml. The results were generally dose related.	The mechanism of this bioassay is not clearly elucidated.
Cell transformation (in vitro)	Curren, et al, 1981 Lewtas, 1983 U.S. EPA, 1983	A series of tests using the Balb/c 3T3 cell line were used to compare a similar set of organic extracts with those used in the enhancement of viral transformation assay. These studies were conducted with and without metabolic activation.	Balb/c 3T3 results were positive for LD diesel and gasoline engines without S9. When tested with S9, only the gasoline emission sample attained statistical significance. Independent tests using the extract of two different LD diesel engines were negative in this bioassay. One HD diesel emission exhaust was negative in this test.	The nature of the data from a quantitative and dose related basis suggests that these results are not easily interpreted.
Skin painting initiation studies in SENCAR mice	Nesnow, et al, 1983 Slaga, et al, 1981 Nesnow, et al, 1982	Each sample was initiated with 5 doses using 40♂ & 40 ♀ (80 mice) per dose. Beginning 1 week after treatment, 2.0 µg TPA was administered twice weekly. Skin tumors were recorded weekly.	Samples from LD diesel engines and LD gasoline engine produced dose-related increases in skin tumors. The maximum activities were observed at dose levels of 1-10 mg/g extract per mouse.	The availability of PAHs may influence these results as samples with higher BAP content showing greater activity. The most potent diesel sample was tested as a complete carcinogen in SENCAR mice and was not active.
Skin tumors at injection site	Pott and Stober, 1983	Subcutaneous injection of automobile exhaust condensate (AEC) were administered to mice in a single injection. The frequency of mice with tumors at the injection site are calculated.	The AEC used in this study was not clearly defined as to origin. The dose levels and tumor incidences were also difficult to evaluate. The results for AEC were clearly positive in this test.	This bioassay is dependent to a large extent on the level of PAH in the test sample.
Co-carcinogenic evaluation of exhaust and diethylnitrosamine (DEN) treatment	Pott and Stober, 1983	Hamsters pretreated with DEN at 1.5 and 4.5 mg/kg BW and then exposed to whole diesel exhaust and to the vapor phase only for 120 weeks. The animals were scored for papillomas of the larynx.	Exposure to whole exhaust and to the vapors increased the larynx papillomas at 4.5 mg/kg DEN but not at 1.5 mg/kg. The vapor component was clearly implicated since whole exhaust did increase the effect further.	The cocarcinogen effect may be the result of SO_2, NO_2 or ozone as well as specific chemicals in the vapor phase.

SELECTION OF BIOASSAYS TO FORM A MINIMUM EVALUATION MATRIX

Objective analysis of the current data base for automobile combustion emissions and test performance criteria has resulted in the selection of three bioassay types which appear to merit further study as screens for automotive emission certification.

The Ames *Salmonella*/microsome reverse mutation assay appears to be useful for several reasons. It responds very sensitively to the mutagens bound to emission particulates especially the nitro-PAHs (Rosenkranz, 1981). The test is flexible and has been used to assess the mutagenic components of the vapor phase of automobile emissions (Stenberg, 1983) and the bioavailability of mutagens in biological fluids (King *et al.*, 1981; Brooks *et al.*, 1981). The data generated by the Ames test meet all quantitative and dose-dependency requirements. Also, the sample size required to perform the test is extremely small compared to that required in other types of bioassays.

Cytogenetic endpoints and especially sister chromatid exchange (SCE) showed very good sensitivity to the genotoxic components of automotive emissions. The tests can be conducted *in vitro* with particle extracts as well as *in vivo* using whole exhaust or only the vapor phase with the expectation of detecting significant responses. The SCE endpoint, although not directly linked to a toxic phenomenon, is perhaps the most versatile and applicable to emission testing among the three tests selected.

The *in vitro* mammalian cell gene mutation assay is the third test considered applicable to emission screening. This determination is based on the assay's quantitative response to organic solvent extracts and, in some cases, to whole unextracted particles (Cheschier *et al.*, 1980; McCormick, 1979). The CHO and Mouse Lymphoma assays generate data from automobile emissions which meet all quantitative and dose-dependency criteria. The endpoint is relevant, but as noted in the detailed analysis of test response, there were a few contradictory responses which should be reviewed and, if possible, resolved prior to developing protocols for test implementation.

Some of the information used to support the selection of these three tests is shown in Table 34 which identifies the range of susceptibilities of various bioassays to the types of samples evaluated. With the exception of Tradescantia and the *in vivo* cocarcinogenicity studies, both of which fail to meet the specifications required for routine application to emission assessment, the three bioassay (endpoint) types selected for emission testing are the only bioassays which demonstrate positive responses beyond organic solvent extract samples. The ability to respond to unextracted particles suggests a higher level of sensitivity of these tests to the possibly relevant genotoxic agents bioavailable from emission particles. While the use of organic solvent extracts may represent the most feasible routine test sample for testing, future methods may rely more upon examination of unextracted particles.

A detailed analysis of the reliability, reproducibility and sensitivity of these bioassays is presented in Part III of this document. This information will provide the basis to interpret test responses from new emission sources for their genotoxic potential.

TABLE 34. DISTRIBUTION OF POSITIVE RESPONSES IN SUSCEPTIBLE BIOASSAYS ACROSS SAMPLE CATEGORIES

Bioassays	Organic Solvent Extracts of Particles	Biological Fluid Extracts of Particles	Unextracted Particles	Vapor Phase Alone	Whole Exhaust
Gene Mutation					
· Microbial	√	(√)		(√)	
· Mammalian Cells In Vitro	√		(√)		
· Plants					
· Insects					
· Mammals In Vivo					
Cytogenetic Endpoints					
· Mammalian Cells In Vitro	√				
· Plants					√
· Mammals In Vivo	√		√		√
Primary DNA Effects					
· In Vitro Mammalian	√				
· In Vivo/In Vitro Mammalian	√				
Carcinogenesis Endpoints					
· Mammalian Cells In Vitro	√				
· In Vivo Mammals (complete)					
· In Vivo Initiation	√				
· In Vivo Co-carcinogenesis				√	√

(√) = data consists of limited experimentation which may not apply to all bioassays of this class.

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PART II

SECTION 4

EFFECTS OF ENVIRONMENTAL CONDITIONS ON BIOLOGICAL ACTIVITY OF EMISSION SAMPLES

EFFECT OF SAMPLING CONDITIONS

The procedure for particulate sampling and measurement involves driving a vehicle or engine on a dynamometer in accordance with Federal Test Procedure driving cycle to simulate typical vehicle speeds and loads. The exhaust is passed into a dilution tunnel connected to a constant volume sampler, where it is mixed with filtered ambient air. The net flow through the tunnel is kept constant (i.e., the sum of exhaust gas and dilution air), to provide proportional sampling. Sampling the diluted exhaust at a constant rate will give an integrated sample representative of a particular driving cycle. The average dilution of the exhaust is generally from 10:1 to 20:1 depending on the engine size and average operating speed. Under highway conditions, the dilution ratio reaches 1000:1 shortly after the exhaust exits the tailpipe. However, as indicated by the investigations of Black and High (1979) and Williams and Chock (1980), the composition of the particulate is fixed when it leaves the tailpipe and is not significantly influenced by further dilution. The data from Williams and Chock (1980) indicate that the B(a)P content tends to increase with dilution while the extractable fraction remains constant (Table 35). No differences were observed in either the particulate emission rates or the molecular weight distribution of the soluble organic fraction (SOF). More recently, studies conducted at the Allegheny Mountain tunnel found the diesel-produced aerosol at Allegheny to be quite similar to the aerosol encountered in dilution-tube experiments with respect to both mutagenicity in the Ames test and molecular-weight distribution (Pierson et al., 1981). Thus, if one hypothesizes that the particle-bound organics are determined primarily during the initial cooling and dilution of the exhaust, the constant-volume-sampling procedure could result in particles chemically and biologically similar to ambient particles.

AMBIENT CONDITIONS

None of these above studies, however, addresses the question of what happens to the particulate organics during prolonged exposure to sunlight and/or to other chemical species present in the atmosphere such as ozone. Ambient conditions can affect the condensation of organic compounds onto particles, influence the interaction between organics, alter the organic species emitted by a source, and provide for various other interactions.

TABLE 35. THE EFFECT OF DILUTION RATIO ON PARTICULATE MASS CONCENTRATION AND COMPOSITION[a]

	Dilution Ratio	No. of Samples	Specific Particulate Concentration ($\mu g/m^3$ particulate/ ppm of CO_2)	Extractables (%)	BaP (ng/mg)
Open Air	<2	1	--[b]	15	5
	5.1-7.0	5	5.0	26	19
	11-17	4	4.1	28	22
	21-24	3	3.5	28	28
	44-88	4	4.5	29	25
	135-150	2	6.5	28	32
	220-350	2	3.7	--[c]	20[d]
Dilution Tunnel	13.8	1	3.9	27	23
	26.5	1	3.8	27	22

[a] Data from Williams and Chock (1980).
[b] CO_2 concentration was above instrument capability.
[c] Extractable mass was too small for accurate determination.
[d] Large background correction to mass makes this number uncertain.

The destruction of polynuclear aromatic hydrocarbons (PAH's) under atmospheric conditions is well known. For example, Falk _et al_. (1956) found that benzo(a)pyrene absorbed on combustion soot was decomposed 10% on exposure to light and air for 48 hours and 18% on exposure to light and smog for only 1 hour. Claxton and Barnes (1981) studied ambient-like conditions using the Calspan smog chamber. Their results indicate that the presence of ozone in the chamber tends to reduce the mutagenic response of the organic material collected, the decrease being most pronounced without metabolic activation.

Model experiments in simulated atmospheres suggest that PAH and other aromatic compounds are transformed in the atmosphere (Pitts, 1982; Nielson _et al_., 1982). The dominant pathways will depend upon seasonal and meteorological conditions, e.g., light intensity, humidity, and temperature, as well as the presence of reactive primary and secondary co-pollutants. Reactions with nitrous acid and sulfur oxides may also occur. At higher solar intensities, photo oxidations as well as reaction with photochemically generated pollutants such as ozone and peroxyacetyl nitrate, may also be important. Photo oxidation reactions transform at least some PAH in model experiments to direct mutagens (McCoy et al., 1979).

EFFECT OF TEMPERATURE

The effect of temperature on emission has been studied by Braddock (1981). In this study, the Federal Test Procedures were conducted at ambient temperatures (average of 12°F above crankcase temperature) without heating of dilution air. The results indicate that decreasing the temperature results in increased particulates and soluble organic fraction (SOF) caused by uncombusted diesel fuel. On a revertant per µg SOF basis, the mutagenic activity without activation appeared to decrease concomitant with decreasing temperature.

ARTIFACT FORMATION DUE TO COLLECTION METHODS

The role of NO/NO_2 during sampling of particles has generated much interest among investigators. It has been demonstrated by various means that mutagenic nitroaromatic compounds are contained in organic extracts of filter-collected particles. Pitts et al. (1978) have shown that nitrated species can be formed under certain experimental conditions. It has also been concluded that the possible formation of nitro-polycyclics is dependent on the filter material (Lee et al., 1980). Basically, nitro-aromatics may be produced in three processes: (1) combustion, (2) exhaust, as organics interact and condense upon the particles, or (3) collection, as an artifact. The possibility of nitration during realistic filter sampling has been investigated. In one experiment, the NO_2 concentration was increased by altering the dilution ratio, by adding NO_2 into the dilution tunnel. An increase in the mutagenic activity was noted at higher concentrations of NO_2 (Bradow, 1980; Claxton, 1980). Fuel aromatics and fuel aliphatics, when exposed to excess NO_2 at 25°C, generated direct acting mutagens for the strain TA-100 (Henderson et al., 1981). Another experiment involved re-exposure of filters previously used. The results obtained with diesel exhausts showed increased levels of 1-nitropyrene and 6-nitro-benzo(a)pyrene as well as heightened mutagenic activity (Gibson et al., 1981). Formation of 1-nitropyrene, a strong mutagen, was shown to have a decisive influence on mutagenicity (Salmeen et al., 1982).

SOLVENT SYSTEMS AND STORAGE METHOD

Hydrocarbons adsorbed on the surface of particulates have been frequently extracted from the particulates and concentrated by using organic solvents in order to assess their specific biological activity. Investigations of solvents representing a wide range of polarities have generally indicated dichloromethane (DCM) to be the most efficient single solvent for extracting mutagens from diesel particulates (Siak et al., 1980, Huisingh et al., 1978). McGrath et al. (1978) tested whole particles suspended in dimethylsulfoxide (DMSO) and obtained negative to moderately positive results for those particles tested using the Ames test. Organic compounds can also be removed by biological fluids, however, the removal process is not nearly so efficient as with DCM or DMSO. King et al. (1981) confirmed that organic solvents are more efficient than physiological fluids in removing mutagens from diesel particles.

Since there is usually a time lapse of several days to several weeks before generated samples can be tested, storage may be an important factor. Huisingh et al. (1978) showed no difference in the mutagenic activity of samples after storage for up to 8 weeks (Figure 8) either on the filter or as an extract. Direct acting components also seem to increase in toxicity with storage. Williams and Chock (1980) conducted a filter-sample-storage study in which eleven filter samples collected from the dilution tunnel were stored in a room at 24° ± 0.5°C and 45%±1% relative humidity with normal fluorescent light. The extractable percentage and B(a)P concentrations were analyzed on randomly selected filters for up to 150 days after collection. Their results show that the total B(a)P loss in 150 days was 57%; B(a)P concentration decreased linearly with time at a rate of 0.046 ng/mg/day, extractable percentage showed no systematic change with storage time (Table 36).

TABLE 36. EFFECT OF FILTER SAMPLE STORAGE ON DIESEL EXHAUST PARTICULATE[a]

Sample Number	Storage Period (days)	Extractable Percentage	B(a)P Concentration (ng/mg)
1	9	34.1	12.1
3	9	32.9	11.1
3	9	32.2	11.1
4	9	32.0	10.9
5	28	38.8	9.8
6	28	39.2	9.0
7	28	40.0	10.1
8	91	44.6	7.7
9	91	40.1	6.3
10	147	36.5	5.5
11	147	37.8	4.3
		37.1 ± 4.0[b]	

[a] Data from Williams and Chock (1980).
[b] One standard deviation

These results suggest that the storage conditions should be carefully controlled to avoid excessive loss of B(a)P by unidentified processes. Good Laboratory Practice would dictate minimizing storage time.

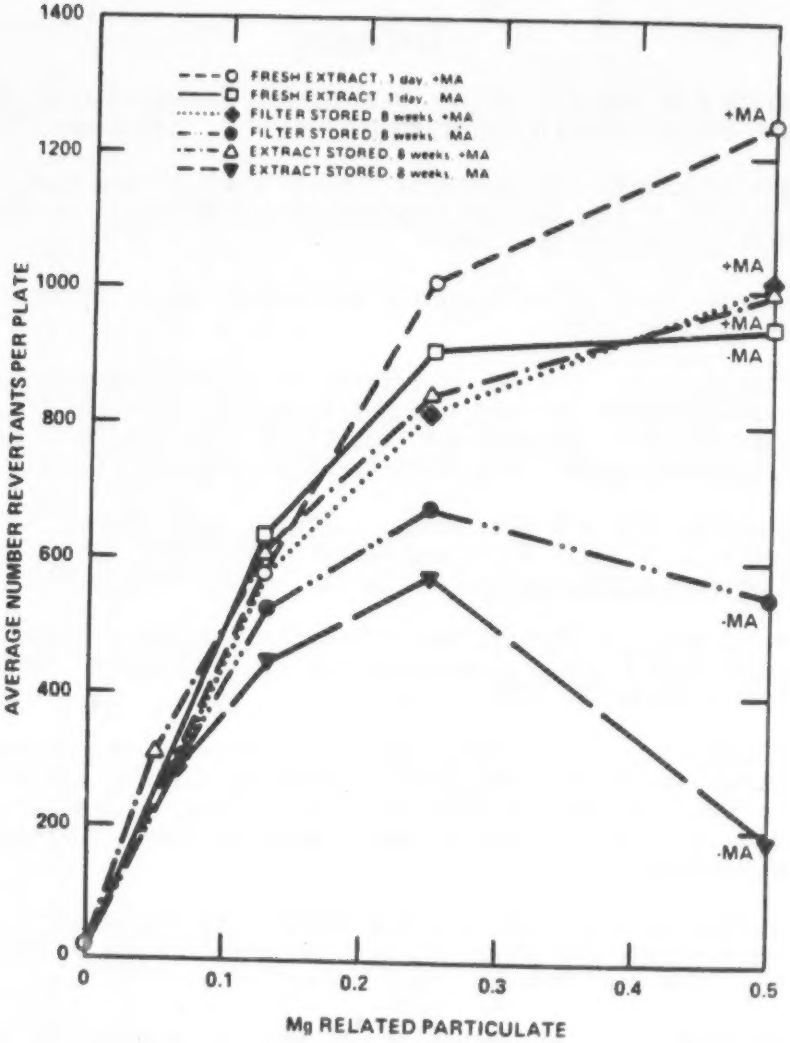

Figure 8. Characterization of diesel particle emissions, Husingh et al., 1978.

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PART II

SECTION 5

EFFECTS OF ENGINE TYPE, FUEL, AND FUEL ADDITIVES ON BIOLOGICAL ACTIVITY OF EMISSION SAMPLES

INTRODUCTION

A variety of factors, including engine design, fuels, lubricants, engine age and operating conditions, may influence the formation of exhaust particulate compounds in exhaust from gasoline- and diesel-powered vehicles (Clark *et al*., 1981; Zweidinger, 1982; and Claxton, 1983). Short-term *in vitro* bioassays have provided a tool for investigating the mutagenic properties of automotive exhausts.

EFFECT OF ENGINE TYPE ON EXHAUST MUTAGENICITY

Comparison of spark-ignition (gasoline)- and diesel-engine-exhaust particle extracts show that gasoline engines produce fewer nitrated PAHs than diesel engines (Nishioka *et al*., 1982; Gibson, 1982). Diesel engines have been reported to produce up to 100 times more respirable particulates per km than gasoline engines with catalytic control. The gaseous emissions of diesel engines contain more NO_x as NO_2, relative to gasoline engines, which contributes to the formation of nitroarenes (Holmberg and Ahlborg, 1983). Diesel vehicles were found to emit about six times as much organic. solvent-extractable particle mass as leaded gasoline vehicles and 20 times more than in-use catalyst cars. Diesel vehicles were also reported to have higher mutagenic emission rates per kilometer than gasoline noncatalyst vehicles, which in turn had higher rates than gasoline catalyst vehicles. Löfroth, *et al*. (1980) also found a higher level of mutagenic components in diesel than in gasoline exhaust: 100 revertants per liter of diesel exhaust compared to 15 revertants per liter of gasoline exhaust in *Salmonella typhimurium* TA98. Zweidinger (1981) reports that the average revertants per mile values (TA98 without S9) were 509,000 for six diesel vehicles; 152,000 for four in-use leaded gasoline vehicles; and 42,000 for 15 unleaded gasoline vehicles. A comparison of three gasoline and six diesel engine equipped cars (Table 37) showed that dichloromethane extracts of particulate exhaust from the gasoline cars were only slightly more mutagenic than those observed for the diesel cars, but the mass of solvent-extractable material on the particulates was lower. Combined with a lower particulate emission rate, this resulted in significantly lower values for revertants per mile for the gasoline-engine-equipped cars (Clark, 1981). This demonstrates the need for normalizing bioassay data for specific needs and comparisons.

TABLE 37. MUTAGENICITY OF SPARK-IGNITION AND DIESEL ENGINE EXHAUST PARTICLE EXTRACTS (Clark, 1981)

Vehicle	Revertants per µg Extract[a]	Extractable Fraction (%)	Extractable Fraction (mg/mi)[b]	Revertants per mile ($\times 10^{-3}$)
Spark Ignition				
Ford Escort	10	8	2	20
Oldsmobile Cutlass	18	5	2	36
Chevrolet Citation	23	5	2	46
Diesel				
Peugeot 504	8	29	60	480
Oldsmobile D-88	12	20	75	900
VW Rabbit	13	26	40	520
Audi 5000	10	43	190	1900
Mercedes 300D	13	13	40	520
Fiat 131	3	71	240	720

[a] Slope of linear portion of dose-response curve in TA 100, without the addition of liver S-9.

[b] Total mass of extractable material from filter ÷ number of miles in test cycle (spark-ignition - 26 miles, diesel - 7.6 miles). The spark-ignition engine cars were operated on unleaded indolene fuel and the diesel cars on a standard No. 2 diesel fuel.

Variability of repeat samples from one car and of samples collected from different identical vehicles is of great concern when evaluating the mutagenicity of mobile-source emissions. Both Claxton and Kohan (1981) and Clark (1981) have investigated the variability associated with multiple sampling of automotive emission.

Variability of repeat sampling on different days of individual automobiles has been shown by Claxton and Kohan and by Clark to vary by less than twofold for all variables compared. Data reported by Clark (Table 38) show that revertants per microgram of extract in three samples collected on different days with each of three different cars were not significantly different ($p>0.01$). Slightly more variability was noted when mutagenic activity was converted to revertants per milligram of particles and revertants per mile. The results demonstrate the repeatability of sample collection, preparation and bioassay.

A comparison of samples from vehicles of the same make, model and operation pattern were shown by Clark (1981) and Claxton and Kohan (1981) to have an increased variability over multiple samples taken from one vehicle. Clark observed that the fraction of extractable organics was more variable with five different diesel equipped Oldsmobile Delta 88 automobiles than observed in triplicate runs with one car of the same type (Table 39).

TABLE 38. VARIABILITY IN MUTAGENICITY OF REPEATED COLLECTIONS OF DIESEL PARTICULATE EXHAUST (Clark _et al_., 1981)

Car	Test No.[a]	Revertants per µg Extract[b]	Extractable Fraction[c]	Revertants per mg Particles	Particle Emissions (g/mi)[d]	Revertants x 10^3 per Mile
VW Rabbit	189	14 (±1.2)	26	3600	0.17	600
	364	15 (±0.6)	29	4200	0.16	700
	192	14 (±1.0)	24	3200	0.19	600
Oldsmobile (#218)	134	12 (±0.7)	22	2500	0.31	800
	280	14 (±1.2)	21	3000	0.36	1100
	283	13 (±1.0)	20	2600	0.38	1000
Oldsmobile (#214)	9896	11 (±0.9)	21	2300	0.29	700
	9892	10 (±0.4)	18	1700	0.38	600
	9893	11 (±0.3)	21	2300	0.36	800

[a] Cars operated according to EPA Federal Test Procedure (hot start) on chassis dynamometer, ambient temperature 75°F. The three sampling periods for each car were on different days.

[b] Slope (± standard deviation) of linear portion of dose-response curve in _Salmonella_ strain TA100 without the addition of a liver enzyme preparation.

[c] Percent (w/w) dichloromethane extractable organics associated with particles.

[d] Grams of particles emitted from exhaust per mile of vehicle operation.

TABLE 39. MUTAGENICITY OF EXTRACTS OF EXHAUST PARTICLES COLLECTED FROM FIVE DIFFERENT OLDSMOBILE DIESEL AUTOMOBILES[a] (Clark _et al_., 1981)

Car-Test No.[b]	Revertants per µg Extract[c]	Extractable Fraction[d]	Revertants per mg Particles	Particle Emissions (g/mi)[e]	Revertants x 10^3 per mile
214-181	10 (±0.9)	18	1800	0.38	700
215-9893	11 (±0.3)	21	2300	0.30	700
216-9942	7 (±0.7)	25	1800	0.33	600
217-284	10 (±0.3)	16	1600	0.34	500
218-252	7 (±0.6)	23	1600	0.33	500

[a] 1980 model year Oldsmobile Delta 88 diesel cars tested after a 4,000 mile break-in period.

[b] Operated according to EPA Federal Test Procedure (hot start) on chassis dynamometer, ambient temperature 75°F.

[c] Slope (± standard deviation) of linear portion of dose-response curve in _Salmonella_ strain TA-100, without the addition of liver enzyme preparation.

[d] Percent (w/w) dichloromethane extractable organics associated with particles.

[e] Grams of particles emitted from exhaust per mile of vehicle operation.

However, the mutagenicity, when normalized to revertants per milligram of particles, was quite similar for each sample, suggesting an inverse correlation between mutagenicity of the extracts and extractable fraction. Claxton and Kohan observed much greater variability in samples taken from five similar gasoline vehicles (Table 40) than seen with either repeat sampling of one vehicle (Table 38) or in comparative testing of similar diesel vehicles (Table 39). There was nearly a tenfold difference in revertants per microgram of organic material among the data from the five cars. As seen with the diesel work, the variability for the bioassay was partially normalized when results were expressed as revertants per gram of particles or revertants per mile. By normalizing the data, variation was reduced from a 10-fold to a 5-fold difference.

Claxton and Kohan also investigated the variation in exhaust particulate mutagenicity among six different light-duty diesel vehicles (Table 41). The variation among different diesel vehicles is similar to variation between the different Ford LTD gasoline automobiles in Table 40 and is also similar to a comparison done by Clark, et al. (1981) with six different light-duty diesel vehicles.

The mutagenicity of exhaust particulates when evaluate in terms of revertants per mile have been seen to vary by two-fold to as much as five-fold in a series of comparitive studies with either gasoline or diesel engines. Repeat sampling of one vehicle produced relatively similar samples that varied less than two-fold in terms of revertants per mile. More variability, up to five-fold difference, was observed when both single samples of identical vehicles and different vehicles within a given class (diesel, gasoline) were compared. This would indicate that collection, processing

TABLE 40. MUTAGENICITY OF EXTRACTS OF EXHAUST PARTICLES FROM FIVE DIFFERENT FORD LTD GASOLINE AUTOMOBILES (Claxton and Kohan, 1981)

Test No.	Car	Slope[a] (Rev/plate/μg)	% Ext.[b]	Rev x 10^5 /g Partic.	PER[c] (g/mile)	Rev/ Mile
0032	Lt. blue	7.54	5.8	4.37	0.0079	3455
0033	Mid. blue	7.51	3.4	2.55	0.0100	2553
0034	Gray	9.49	3.2	3.04	0.0180	5466
0035	Silver	9.61	3.8	3.65	0.0100	3652
0036	Brown	1.02	21.4	2.18	0.0050	1091
Mean		7.03	7.52	3.16	0.0102	3243
Standard dev.		3.51	7.83	0.87	0.0048	1602
Coeff. of var.		0.50	1.04	0.28	0.47	0.49

[a] Slope of linear regression line.
[b] Percent dichloromethane-extractable mass.
[c] Particle emission rate.

TABLE 41. MUTAGENICITY OF EXTRACTS OF EXHAUST PARTICLES COLLECTED FROM SIX DIFFERENT DIESEL VEHICLES (Claxton and Kohan, 1981)

Vehicle	Slope[a] (Rev/plate/µg)	Mean % Ext.[b]	Rev x 10^5 /g Partic.	PER[c] (g/mile)	Rev x 10^5 /mile
Olds 350	1.85	21.1	3.90	0.846	3.30
Olds 260	1.51	16.5	2.49	1.005	2.50
Chev. Truck 350 diesel	2.64	53.4	14.10	0.623	8.78
M.B. 300D	3.23	23.3	7.53	0.840	6.32
Opel Record E	1.15	51.6	5.93	0.483	2.87
VW Dasher	1.45	53.7	7.79	0.322	2.51
Mean	1.97	36.6	6.96	0.687	4.38
Standard deviation	0.80	18.0	4.06	0.256	2.59
Coefficient of variaion	0.41	0.49	0.58	0.37	0.59

[a] Slope of linear regression line.
[b] Percent dichloromethane-extractable mass.
[c] Particle emission rate.

and bioassay of automotive particulate emission samples can be standardized to produce reproducible results. Results also indicate that sample variation may be expected to arise from either operating characteristics of a particular test vehicle or from basic differences in combustion related to engine design. The most dramatic difference observed of the effect of engine type on exhaust mutagenicity was the difference between gasoline and diesel engines. When normalized to revertants per mile, both Claxton and Kohan (1981) and Clark et al. (1981) observed a 50- to 100-fold higher level of mutagenicity with diesel exhaust particulate than with gasoline exhaust particulate.

EFFECT OF FUELS ON EXHAUST MUTAGENICITY

Many variables have been shown to influence particulate exhaust emission rates of diesel engines. These include fuel density, aromaticity and distillation properties, fuel additives, and make and model of car (Clark et al., 1982a; Hare, et al., 1978; Springer and Baines, 1977; Lipkea, et al., 1978; Burley and Rosebrock, 1979). The correlation between fuel composition and mutagenicity of diesel exhaust particulates has not been so clearly established. Several studies have investigated the correlation between fuel aromatic content and mutagenicity of organic extracts of exhaust particulate material (Zweidinger, 1982; Sklarew et al., 1981; Clark et al., 1982a). Generally, no well-defined relationship between aromatic content of the fuel and mutagenicity of the exhaust particle extract was noted. Clark et al., suggested that the relative abundance of various PAH species may be important since higher boiling fuels generally produced more mutagenic combustion products than lower boiling fuels. However, Zweidinger (1982) investigated several fuel parameters and their correlation with mutagenic activity (revertants per microgram of extract). The highest correlations noted were associated with nitrogen content of the fuel. Subsequent studies of the effect of nitrogen levels (obtained by doping a base fuel with isoquinoline) did not show any correlation between mutagenicity and fuel nitrogen level. Slight correlation was reported between aromaticity and activity, particularly without activation.

In investigating the effect of aromatic content on exhaust particulate mutagenicity, Clark et al. (1982a) burned a variety of fuels (with aromatic content ranging from 34 to 13 percent) in two different vehicles. All samples were mutagenic in TA-100 without metabolic activation. However, the data, summarized in Tables 41 and 42, do not show a clear-cut relationship between mutagenicity of dichloromethane extracts or revertants per mile and fuel aromatic content. For the Oldsmobile, higher aromatic fuels gave particle extracts with higher mutagenic potency but the mass of extractable material was lower (Table 42). In the Peugeot, higher aromatic content fuels did not result in the lower particle extract masses as seen in the Oldsmobile (Table 43). Also, there was no large difference in particle mutagenicity and revertants per mile as noted with some Oldsmobile fuel tests. These results suggest an influence of engine design on the production of mutagenic particle extracts.

Clark reported further evidence that mutagenicity is not directly related to fuel aromatic content. An Oldsmobile was operated on three fuels, ranging from 32 to nearly 0 percent aromatics. As can be seen in Table 44, high aromaticity was associated with high particle emission rates but lower extractable particulate fraction. The isoparaffin fuel of almost 0 percent aromatic content had higher mutagenicity than the jet fuel with 17 percent aromatics.

TABLE 42. MUTAGENICITY OF EXTRACTS OF PARTICLES COLLECTED FROM THE EXHAUST OF AN OLDSMOBILE DIESEL CAR[a] OPERATED ON DIFFERENT FUELS (Clark et al., 1982a)

Fuel[b]	% Aromatics	Revertants per μg Extract[b]	Extractable Fraction (%)[c]	Revertants per mg Particles	Particle Emissions (g/mi)[d]	Revertants per Mile (x 10^3)
7943	34	10 (0.4)	22	2200	0.22	500
7926	32	18 (1.2)	21	3800	0.31	1200
7940	32	8 (0.5)	22	1800	0.25	400
7942	20	7 (0.2)	41	2900	0.23	700
7939	20	5 (0.4)	25	1300	0.21	300
7938	13	7 (0.4)	30	2100	0.22	500
7941	13	7 (0.3)	30	2100	0.24	500

[a] 1980 model year Oldsmobile Delta 88 (5.7 liter) operated according to EPA Federal Test Procedure (hot start) on a chassis dynomometer, ambient temperature 75°F.

[b] Slope (± standard deviation) of linear portion of dose-response curve in *Salmonella* strain TA-100, without the addition of Liver S-9.

[c] Percent (w/w) dichloromethane extractable residue associated with particles.

[d] Grams of particles emitted from exhaust per mile of vehicle operation.

TABLE 43. MUTAGENICITY OF EXTRACTS OF PARTICLES COLLECTED FROM THE EXHAUST OF A PEUGOT DIESEL CAR[a] OPERATED ON DIFFERENT FUELS (Clark et al., 1982a)

Fuel[b]	% Aromatics	Revertants per μg Extract[b]	Extractable Fraction (%)[c]	Revertants per mg Particles	Particle Emissions (g/mi)[d]	Revertants per Mile (x 10^3)
7943	34	4 (0.3)	17	700	0.18	100
7926	32	7 (0.2)	32	2200	0.20	400
7940	32	6 (0.2)	30	1800	0.17	300
7942	30	3 (0.3)	23	700	0.18	100
7939	20	7 (0.2)	22	1500	0.23	400
7941	13	4 (0.3)	18	700	0.17	100

[a] 1979 Model year Peugeot 504 (2.3 liter) operated according to EPA Federal Test Procedure (hot start) on a chassis dynomometer, ambient temperature 75°F.

[b] Slope (± standard deviation) of linear portion of dose-response curve in *Salmonella* strain TA-100, without the addition of Liver S-9.

[c] Percent (w/w) dichloromethane extractable residue associated with particles.

[d] Grams of particles emitted from exhaust per mile of vehicle operation.

TABLE 44. INFLUENCE OF A JET "A" AND ISOPARAFFINIC FUELS ON MUTAGENICITY OF EXHAUST PARTICLE EXTRACTS OF AN OLDSMOBILE DIESEL CAR[a] (Clark, et al., 1982a)

Fuel[b]	Revertants per μg Extract[c]	Extractable % Fraction[d]	Revertants per mg Particles	Particle Emissions (g/mi)[e]	Revertants per Mile (x 10^3)
Reference (32%)	13	23	2900	0.33	950
Jet "A" (17%)	5	25	1200	0.20	240
Isoparaffin (0.4%)	8	40	3000	0.16	480

[a] 1980 Model Oldsmobile Delta 88 (5.7 liter) operated according to EPA Federal Test Procedure (hot start) on a chassis dynomometer, ambient temperature 75°F.

[b] Reference Fuel - 7926; Jet "A" fuel - 8017; isoparaffin fuel - 7812; % aromatic content in parentheses.

[c] Slope (± standard deviation) of linear portion of dose-response curve in Salmonella strain TA-100, without the addition of liver S-9.

[d] Percent (w/w) dichloromethane extractable organics associated with particles.

[e] Grams of particles emitted from exhaust per mile of vehicle operation.

Chemical analysis of extracts of exhaust particulates indicates that mutagenic direct-acting nitroaromatic compounds may be responsible for much of the observed mutagenic activity. It has been suggested that nitroaromatics, such as nitropyrenes and nitronaphthalenes, are normal components of diesel and even some gasoline exhaust (Schuetzle et al., 1982; Nishioka et al., 1981). These nitroaromatics may be caused partially by reactions of unburned fuel PAH and NO_x formed in the combustion process (Henderson et al., 1981); or they may be associated with particulate exhaust material contributed by the engine oil (Mayer et al., 1980).

The definitive relationship between the mutagenicity of particulate-associated organic material produced by combustion and aromatic content of fuels has not been established. Aromatic content may be influential at high aromatic levels (>30%), but appears to depend upon engine type. Because of the variability in diesel fuel composition associated with the variety of crude oils and refining techniques, control of fuel properties to affect emission mutagenicity may be a futile strategy (Seizinger et al., 1982).

The addition of fuel extenders has been shown to affect the emission characteristics of diesel fuels (Clark et al., 1982b). Addition of butanol, ethanol or middle distillate fraction of SRC-II (a solvent refined coal liquid) to diesel fuel decreased the specific mutagenicity of dichloromethane extracts of diesel exhaust particles, but increased the particle-associated organic material two- to four-fold. This resulted in higher mutagenicity (revertants per mile) using the three extenders than using diesel fuel alone. Rannug (1983) found comparable mutagenicity in acetone extracts of particulates collected from engines powered by leaded gasoline, unleaded gasoline, or a lead-free gasoline with 15 percent methanol. In studies of acetone extracts of particles, he noted that three distinct mutagenic groups were formed depending upon the fuel used. Using Ames data with TA98 and TA100, light-duty diesel vehicles formed a high mutagenicity group giving between 50-250 revertants/L of exhaust or 100,000 to 700,000 revertants/km. The second group contained the engines fueled by leaded and lead-free gasoline as well as gasoline-alcohol fuels; these gave 10-50 revertants/L of exhaust or 10,000 to 20,000 revertants/km. The final group of fuels, methanol and propane gave less than 10 revertants/L or less than 20,000 revertants/km.

MUTAGENICITY OF CRUDE OILS AND FUELS

Mutagenicity of diesel and gasoline engine emissions would indicate that the mutagenic components occur in the fuel or are synthesized at some point in the combustion/exhaust cycle. A variety of crudes and their fractions have been tested for mutagenic activity. Huisingh et al. (1978) reported that fractions of a diesel fuel were not found to be mutagenic, suggesting that mutagens detected in the exhaust were products of the combustion process itself. Lebowitz et al. (1979) reported diesel fuel as non-mutagenic in a battery of short-term assays. Wang et al. (1978a, 1978b) noted that a diesel fuel, JP-4, and two types of gasoline were not mutagenic with TA-98 in the Salmonella bioassay.

Some investigators have found some heavier crude oils including petroleum-, coal- and shale-derived distillates to be mutagenic. Synthetic (shale-derived) oils have been shown to have mutagenic activity in Salmonella (Epler et al., 1979; Pelroy and Petersen, 1979; Rao et al., 1978). Guerin et al. (1978), Epler et al. (1978) and Ellenton et al. (1981) found fractions of natural crudes to have mutagenic activity in Salmonella. When fractionated, they found that mutagenic activity was located in the PAH-containing (neutral) fraction. Epler et al. (1978) and Guerin et al., (1980) showed that a coal-derived petroleum substitute was ten times more mutagenic than a petroleum-derived product. Additionally, they reported that activity of natural petroleum crudes was located mainly in the neutral fractions, while significant activity was found in both the neutral and basic fractions of coal-derived fuels.

MUTAGENICITY OF LUBRICATING OILS

Crankcase oils from both diesel and spark-ignition vehicles have been studied for their mutagenic components and for the contribution lubricating oils make to mutagenic components in vehicular emissions. Unused crankcase oils have been reported to be non-mutagenic in the Salmonella bioassay, but used crankcase oils became mutagenic in gasoline engines (Payne et al., 1978; Wang et al., 1978; Hermann et al., 1980; Löfroth et al., 1980). Löfroth reported that this mutagenic activity increased in proportion to the number of kilometers driven and, with TA-98 that metabolic activation increased the mutagenic activity of used motor oil. Used motor oil from diesel engine gave small increases in the reversion frequency in the Salmonella assay but the effect was not dose-dependent (Löfroth et al., 1980).

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PART III

SECTION I

SELECTION OF BIOASSAYS FOR ROUTINE SCREENING OF AUTOMOBILE EMISSIONS

INTRODUCTION

The first two parts of this document addressed the issues of whether short-term tests were amenable to the assessment of auto emission genotoxicity and if so what has been the experience of the specific tests in this regard.

The conclusions indicated that, for several reasons, short-term tests would be extremely useful in the assessment of mutagenic and/or carcinogenic potential. Among the numerous tests which have been used to evaluate the genotoxicity of automobile emissions, three appear to offer the best assessment battery. They are: Ames *Salmonella*/liver microsome assay, Sister Chromatid Exchange (SCE) analysis in Chinese Hamster Ovary (CHO) cells, and tests for gene mutation in cultured mammalian cells (particularly the Mouse Lymphoma assay). This latter conclusion, of course, is based upon an analysis of the current data. As the data base expands, it may become evident that other assays provide an improved assessment; thus, the utility of the test battery selected should be periodically reviewed with this possibility in mind.

The three tests identified as those currently considered to be most useful in emission evaluation have also been identified as predictive tests for human health effects, in particular, cancer initiation. The reliability of these three tests in combination in predicting animal carcinogenicity is very good (Bartsch *et*. *al*., 1983; Clive *et* *al*., 1979; Wolff, 1983). The reliability of these tests or animal cancer studies to predict human cancer risk is limited by the available human epidemiology data base (see Table 11).

PROCEDURES

The Office of Pesticides and Toxic Substances (OPTS) of the EPA has published guideline protocols for each of the candidate screening bioassays identified. Copies of these protocols are available through the National Technical Information Service (5285 Port Royal Road, Springfield, Virginia 22161). Each protocol has been subjected to peer review and meets the minimal criteria for a screening procedure. However, several components of these protocols require modification if they are to be adapted to emission screening.

1. Emissions exist in multiphase forms (gas, solid, extractable) which necessitate that sample collection and preparation information unique to the type emission assessment be added to the protocol. At the present time, it is recommended that analyses be limited to extracts of the particle phase of the emission.

2. The analysis of test sample results should go beyond qualitative evaluations of activity or nonactivity. The primary usefulness of this approach will rely upon quantitative comparative assessments among samples. The experimental results from numerous tests of engines, running conditions, and fuel types indicate that quantitative variation among positive responses will be involved in decisionmaking. Few, if any, combustion samples are inactive when evaluated as particle extracts. Therefore, techniques for quantitative analysis and comparison among samples should be included in the study designs.

Section 2 of Part III of this report will be devoted to these two parameters.

DATA INTERPRETATION

Interpretation of test results in a manner that would permit the establishment of minimal criteria for engine or fuel assessment will be the most difficult issue to resolve. An approach is one in which bioactivity ceilings (limits) are established. These activity limits will not necessarily reflect safety factors because problems associated with adverse health consequences of automobile emissions have yet to be resolved; however, the limits must be established on the basis of the best available information obtained from published reports.

A secondary parameter of this problem is one of data format (e.g., expression of the data in constant units). Ames test results are often converted to revertants per mile (or kilometer) or revertants per unit of fuel combusted. While adequate for such a test, these conversions would not necessarily work equally well for SCE or quantitative mutation frequencies generated in mouse lymphoma L5178Y or other cell lines.

Problems of data interpretation and standardized criteria for engine and/or fuel certification will be addressed in Section 3 of this part of the report.

POTENTIAL PROBLEMS

While the prospects for using short-term bioassays in the evaluation of exhaust emissions look promising based on the published literature, other technology related issues must be addressed. Claxton summarized these issues in a report published in 1982. A summary of these pertinent issues is given in Table 45.

TABLE 45. DIFFICULTIES ENCOUNTERED IN THE INTERPRETATION OF MOBILE SOURCE GENOTOXICITY STUDIES

Stage	Type of Difficulty
Generation	Characterization of the source; Repeatability of testing procedure; Vehicle-to-vehicle variation.
Collection	Artifact formation (NO_x, organic interaction, ozone); Control versus ambient conditions.
Extraction	Physiological versus organic; Selective loss and recovery of compounds; Artifact formation.
Fractionation	Recovery efficiencies; Complexity.
Bioassay	Relevance; Reproducibility; Endogenous activation; Bioassay versus human metabolism.
Statistical	Collection/recovery efficiencies; Data transformations; Data summary methods; Statistical/correlation methods.

From: Claxton, L.D. The utility of bacterial mutagenesis testing in the characterization of mobile source emissions: A review. In: 1981 Diesel Emissions Symposium. U.S. Environmental Protection Agency. pp. 81-94, 1982.

PART III

SECTION 2

TEST PROCEDURES

PROTOCOL RECOMMENDATIONS

Copies of protocols for the Ames test, SCE test using CHO cells, and for specific locus gene mutation in Mouse Lymphoma L5178Y (TK+/-) cells* and Chinese Hamster Ovary (CHO) (HGPRT+)** cells are attached. The only procedural comment regarding the conduct of these tests relates to the fact that each should be conducted in such a manner that:

1. Dose levels will cover toxic as well as nontoxic concentrations of the sample.

2. Sufficient dose levels will be used to enable a multipoint dose response curve to be constructed for each test condition employed.

3. Exposures will be calibrated in a manner that will permit data to be expressed as induced events per unit of emission released per mile traveled or mass of fuel consumed.

4. Each assay should be calibrated against a standard sample (i.e., standard diesel particulate, standard gasoline particle). The standardization would consist of periodic inclusion of an extract of the standard sample in the bioassay. A dose-response curve following within a specified range would be mandatory before contigued testing of routine samples. Also, it would be highly advantageous to include a single dose from the response curve of the standard sample in each bioassay set conducted on a given day or with a given cell pool.

5. It is recommended, to fulfill the data requirements for quantitative comparisons, that each bioassay be conducted with two replicates and repeated once, no earlier than 24 hours following the first trial.

*Five specific assay acceptance criteria for deciding whether an assay is usable have been developed by NTP under their testing program.

**Use of CHO cells should be limited to the modified exposure method reported by Li et al., 1982.

RECOMMENDATIONS FOR COLLECTION AND PROCESSING OF SAMPLES

Ideally, one would like to be able to compare engines or fuels using whole-exhaust emissions under conditions similar to human exposure. Experience to date, however, indicates that the ideal is probably not feasible with current state-of-the-art techniques. The best situation would be to evaluate the fractionated whole exhaust (i.e., gas phase and solid phase separately). This requires that the whole exhaust be trapped and specially processed for in vitro bioassay procedures. Part II, Section 1 describes many of the sampling procedures applied to exhaust emissions. The dilution tunnel method has worked well and can be adapted to both gas phase and particle trapping. At the present time, either the absorbant resin or cryogenic trapping of gas phase materials would appear adequate, although both have significant limitations and additional research into the best method for gas phase evaluation is needed. In situ methods may also be of value.

Part II, Section 2 describes the techniques for preparing collected particulates for bioassay. The typical process involves dichloromethane soxhlet extraction followed by solvent exchange to DMSO. This procedure is applicable to all three of the candidate bioassays. While particle size and loading properties might vary slightly as fuels or engines vary, for the purposes of comparison, these factors as well as differences in bioavailability from particle to particle will not be considered, and extracts prepared under uniform conditions will be compared for potency.

It may be possible at some point to develop a reliable bioassay responsive to the toxic properties of whole exhaust but, until that time, the most efficient and reproducible method consists of dilution tunnel trapping (particulates and/or gases), extraction with organic solvents, and quantification of the total organics collected followed by solvent exchange and the selected bioassay(s).

This process is shown in a general manner in Figure 9 and should be used to form the basic steps of a routine testing program.

RECOMMENDATIONS FOR DATA ANALYSIS

Each protocol previously recommended contains a series of criteria for the determination of a positive response. In all cases, this involves a statistical analysis of the data. Because of the use of these tests in a program for engine or fuel assessment, the analysis should be more extensive and should synthesize the responses of the three candidate screening procedures into the final determination of genotoxic activity.

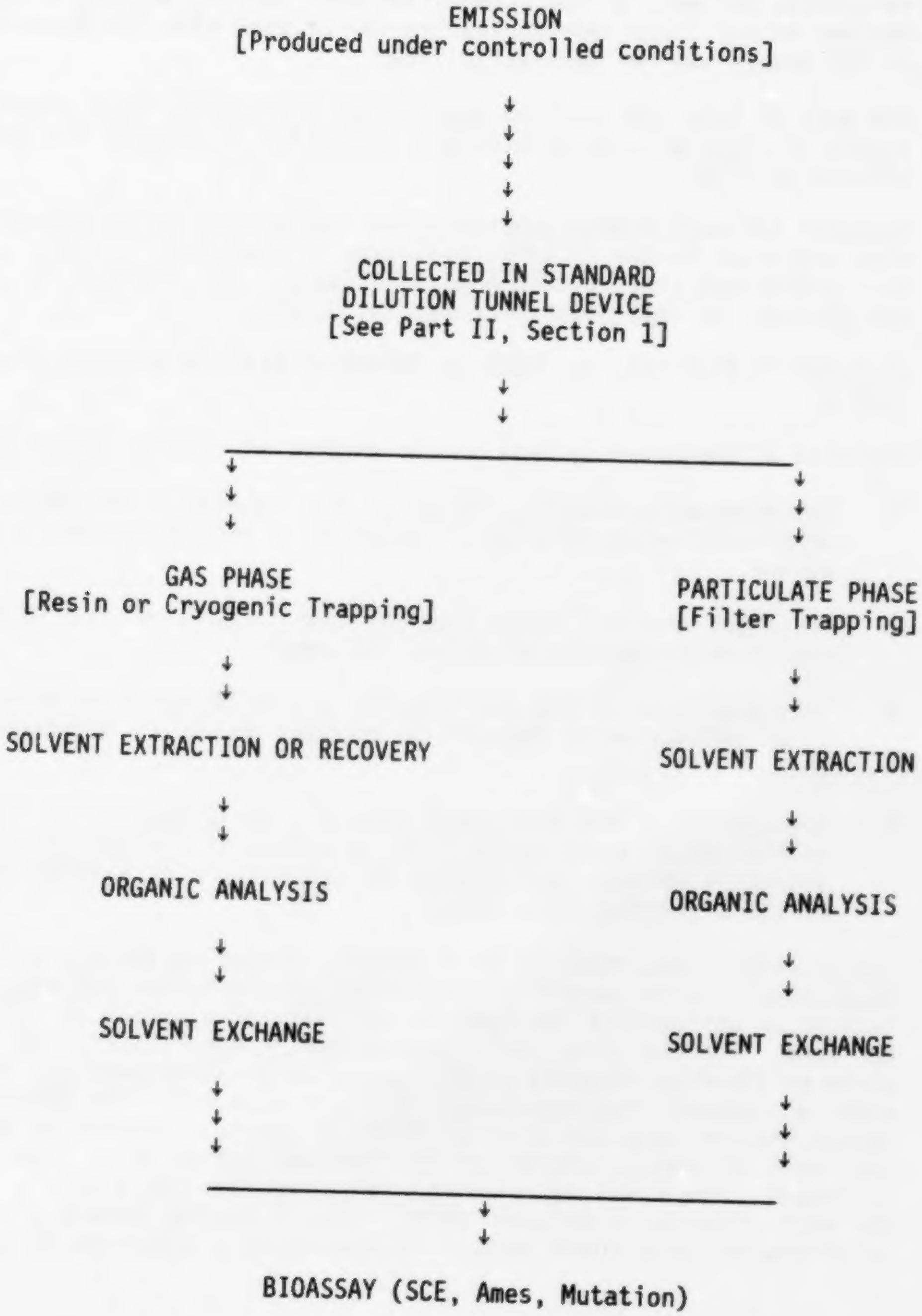
EMISSION
[Produced under controlled conditions]
COLLECTED IN STANDARD DILUTION TUNNEL DEVICE
[See Part II, Section 1]
GAS PHASE
[Resin or Cryogenic Trapping]
PARTICULATE PHASE
[Filter Trapping]
SOLVENT EXTRACTION OR RECOVERY
SOLVENT EXTRACTION
ORGANIC ANALYSIS
ORGANIC ANALYSIS
SOLVENT EXCHANGE
SOLVENT EXCHANGE
BIOASSAY (SCE, Ames, Mutation)

Figure 9. Flow Chart for Sample Collection and Processing.

Data from the Ames test have been subjected to numerous statistical evaluation, potency assessment (Russell and Meselson, 1977; Bartsch, 1983; Hooper _et al_., 1977) and normalization (i.e., revertants/ km; revertants per mass of fuel combusted) with varying degrees of success. Whether or not these same approaches can be used with the Mouse Lymphoma or SCE assays has not been established.

One must at this time caution against any presumption that potency of a sample _in vitro_ will be an accurate indication of potency for health effects _in vivo_.

Probably the most useful generalization reflecting the relevance of positive responses in the _in vitro_ bioassays is that they indicate a potential for hazard, and that, _within_ a given bioassay, the stronger the response, the greater the indication of potential hazard.

APPROACH TO DATA ANALYSIS USED IN COMPARING GENETIC ACTIVITY OF EMISSION SAMPLES

Analysis of the bioassay data should accomplish several objectives:

1. Interpretation should be based on test results expressed in a manner which will relate biological activity to emissions rate in a useful manner.
2. Responses from all three bioassay types should be used in evaluating the genetic toxicity of a specific sample.
3. Interpretation of the test results should be based on sound statistical methods which capture the maximum amount of information available.
4. Analysis of a specific sample should clearly demonstrate the degree of biological activity relative to a body of similar samples and determine whether that biological activity falls outside the range of the historical data base.

The procedures described in this section attempt to satisfy each of the objectives. To be useful, a description of biological activity must include an estimate of the specific activity of a sample in each test employed. For the three tests recommended as the battery, the linear slope of the dose-response curve, expressed as revertants/μg, SCE/cells/μg/ml and mutant frequency/μg/ml, has been selected. The specific activity should then be adjusted to an estimate of particle potency by determining the level of organic extractability (percent extractable) from the material collected. The final calculation should consider the particle potency _vs_. the rate of particle emission (PER). The following formula gives the relationship among these factors in developing a Sample Activity Rate (SAR).

$$\underbrace{\underset{\substack{\uparrow\\ \text{Slope}\\ \text{(Specific Activity)}}}{\frac{\text{Genetic Activity}}{\mu\text{g organics}}} \times \underset{\substack{\uparrow\\ \text{\% Extractables}}}{\frac{\mu\text{g organics}}{100\ \mu\text{g particles}}}}_{\text{Particle Potency}} \times \underset{\substack{\uparrow\\ \text{Particle Emission}\\ \text{Rate (PER)}}}{\frac{\mu\text{g particles}}{\text{kilometer (km)}}} = \underset{\substack{\uparrow\\ \text{Sample Activity}\\ \text{Rate (SAR)}}}{\frac{\text{Genetic Activity}}{\text{km}}}$$

The SAR developed for the three bioassays selected would be expressed in the following manner:

Test	SAR Format
Ames	Revertants/km
Mouse Lymphoma	Mutant Frequency/km
SCE	SCE/km

The proposed approach allows all factors contributing to the genetic activity of a sample to be converted to a constant unit of emission release. No attempt is implied to associate these values with any quantitative estimate of genetic or carcinogenic risk.

Table 44 identifies a set of samples obtained by the EPA (HERL/RTP) and tested in the three candidate bioassays. Different engines and fuels are represented in this sampling and, in many respects, this group of samples represents a realistic cross-section of data likely to be encountered in routine emission evaluation.

Under the proposed testing scheme, future test samples will be accompanied by more data from independent trials; thus, estimates of variability will be more accurate.

SAR for the 14 samples were calculated for the Ames test (Table 46), Mouse Lymphoma assay (Table 47), and SCE assay (Table 48). Values for nonactivation and activation trials are shown. Further analysis of the data sets resulted in the conclusion that S9 mix did not significantly alter the SAR and all subsequent analyses of the data treated nonactivation and activation runs as equivalent.

Objectives No. 2 through No. 4 necessitate application of statistical methods which would identify the samples that produced responses in one or more of the selected bioassays which fell outside a "normal" range of biological activity.

TABLE 46. DESCRIPTION OF SOURCES FOR FOURTEEN EMISSION SAMPLES COMPARED FOR MUTAGENIC ACTIVITY

Sample No.	Engine (Fuel)	Sample No.	Engine (Fuel)
2	1970, 6 cylinder Ford Van (leaded gas)	12	GM DD 8V-71 Diesel (#2 diesel fuel, Prem.)
3	1979, 300D Mercedes Diesel (#2 diesel fuel)	13	GM DD 8V-71 Diesel (#1 diesel fuel)
4	Caterpillar 3208 Diesel (#2 diesel fuel, Ave.)	14	GM DD 8V-71 Diesel (Jet fuel)
5	Caterpillar 3208 Diesel (#2 diesel fuel, Min.)	16	Oldsmobile 350 (#2 diesel fuel, Min.)
6	Caterpillar 3208, Diesel (#2 diesel fuel, Prem.)	17	Mustang II (unleaded gasoline)
10	GM DD 8V-71 Diesel (#2 diesel fuel, Ave.)	18	Nissan Diesel 220C (#2 diesel fuel, Min.)
11	GM DD 8V-71 Diesel (#2 diesel fuel, Min.)	19	Volkswagon Rabbit (#2 diesel fuel, Min.)

The approach which was selected employed an analysis of variance which compared the experimental means at the 0.05 level of significance for each sample in each bioassay. The sample means are plotted in tables for comparative purposes (Figures 10-12). All data from a given bioassay were included in an analysis because there was no evidence which indicated a significant S9 effect and no evidence which indicated an interaction effect.

Several methods may be used to ask the question of how to identify the outliers among the samples in Figures 10-12. For example, it is possible to group samples using various statistical methods and find those tests which differ significantly from the remaining tests. Samples 5 and 18, for example, are clearly outliers using the Ames test (Figure 10).

TABLE 47. TIER STUDY - AMES TEST - TA98
ANALYSIS OF REVERTANTS PER KILOMETER

Sample	ACT[1]	Slope[2]	PER[3] μg/km	% EXT[4]	REV. Per KM[5]
2	2	6.630	0.0300	18.7	37.194
2	1	5.110	0.0300	18.7	28.667
3	2	2.380	0.2400	8.4	47.981
3	2	4.380	0.2400	8.4	88.301
3	1	5.400	0.2400	8.4	108.864
3	1	2.040	0.2400	8.4	41.126
4	2	1.020	0.5600	53.9	307.877
4	2	1.050	0.5600	53.9	316.932
4	1	0.900	0.5600	53.9	271.656
4	1	1.040	0.5600	53.9	313.914
5	2	1.760	0.5900	58.7	609.541
5	2	1.500	0.5900	58.7	519.495
5	1	1.340	0.5900	58.7	464.082
5	1	1.300	0.5900	58.7	450.229
6	2	0.940	0.5500	56.8	293.656
6	2	0.800	0.550	56.8	249.920
6	1	1.070	0.5500	56.8	334.268
6	1	0.830	0.5500	56.8	259.292
10	2	0.045	2.0700	17.5	16.301
10	2	0.124	2.0700	17.5	44.919
10	1	0.043	2.0700	17.5	15.577
10	1	0.081	2.0700	17.5	29.342
11	2	0.207	1.9400	17.7	71.080
11	2	0.233	1.9400	17.7	80.008
11	1	0.184	1.9400	17.7	63.182
11	1	0.199	1.9400	17.7	68.333
12	2	0.102	1.9600	18.2	36.385
12	2	0.120	1.9600	18.2	42.806
12	1	0.068	1.9600	18.2	24.257
12	1	0.053	1.9600	18.2	18.906
13	2	0.081	1.3600	23.1	25.447
13	2	0.090	1.3600	23.1	28.274
13	1	0.039	1.3600	23.1	12.252
13	1	0.037	1.3600	23.1	11.624
14	2	0.118	1.2200	29.5	42.468
14	2	0.074	1.2200	29.5	64.062
14	1	0.074	1.2200	29.5	26.633
14	1	0.051	1.2200	29.5	18.355
16	2	1.500	0.8400	17.0	214.200
16	1	2.200	0.8400	17.0	314.160
17	2	3.500	0.0085	43.0	12.792
17	1	1.600	0.0085	43.0	5.848
18	2	13.000	0.5300	8.0	551.200
18	1	11.000	0.5300	8.0	466.400
19	2	3.000	0.2900	18.0	156.600
19	1	3.800	0.2900	18.0	198.360

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[1] Activation conditions are: ACT 1 = -S9; ACT 2 = +S9.
[2] Slope of linear portion of dose response curve.
[3] Particle Emission Rate.
[4] Percent of total particle weight extracted.
[5] SAR (Revertants/KM) Sample Activity Rate.

TABLE 48. TIER STUDY - MOUSE LYMPHOMA ANALYSIS OF MUTATION FREQUENCY PER KILOMETER

Sample	ACT[1]	Slope[2]	PER[3] µg/km	% EXT[4]	MF Per KM[5]
2	2	5.742	0.0300	18.7	32.213
3	2	1.502	0.2400	8.4	30.280
4	2	0.280	0.5600	53.9	84.515
4	1	0.327	0.5600	53.9	98.702
5	2	0.389	0.5900	58.7	134.722
5	1	0.988	0.5900	58.7	342.174
6	2	0.279	0.5500	56.8	87.160
6	1	0.274	0.5500	56.8	85.598
11	2	1.257	1.9400	17.7	431.629
12	2	0.547	1.9600	18.2	195.126
13	2	1.171	1.3600	23.1	367.881
13	1	0.513	1.3600	23.1	161.164
14	2	0.143	1.2200	29.5	51.466
14	1	0.052	1.2200	29.5	18.715
16	2	1.280	0.8400	17.0	182.784
16	1	1.210	0.8400	17.0	172.788
17	2	1.090	0.0085	43.0	3.984
17	1	0.380	0.0085	43.0	1.389
18	2	2.870	0.5300	8.0	121.688
18	1	4.190	0.5300	8.0	177.656
19	2	0.720	0.2900	18.0	37.584
19	1	0.980	0.2900	18.0	51.156

[1] Activation conditions are: ACT 1 = -S9; ACT 2 = +S9.

[2] Slope of linear portion of dose response curve.

[3] Particle Emission Rate.

[4] Percent of total particle weight extracted.

[5] SAR (Mutant Frequency/KM) Sample Activity Rate.

TABLE 49. TIER STUDY - SISTER CHROMATID EXCHANGE ANALYSIS OF EXCHANGES PER KILOMETER

Sample	ACT[1]	Slope[2]	PER[3] 3 μg/km	% EXT[4]	SCE Per KM[5]
2	1	0.614	0.0300	18.7	3.4445
2	2	0.465	0.0300	18.7	2.6086
3	1	0.091	0.2400	8.4	1.8346
3	2	0.160	0.2400	8.4	3.2256
4	1	0.010	0.5600	53.9	3.0184
4	2	0.038	0.5600	53.9	11.4699
5	1	0.156	0.5900	58.7	54.0275
5	2	0.061	0.5900	58.7	21.1261
6	1	0.214	0.5500	56.8	66.8536
6	2	0.065	0.5500	56.8	20.3060
10	1	0.025	2.0700	17.5	9.0562
10	2	0.048	2.0700	17.5	17.3880
11	1	0.081	1.9400	17.7	27.8138
11	2	0.021	1.9400	17.7	7.2110
12	1	0.032	1.9600	18.2	11.4150
12	2	0.034	1.9600	18.2	12.1285
13	1	0.050	1.3600	23.1	15.7080
13	2	0.032	1.3600	23.1	10.0531
14	1	0.017	1.2200	29.5	6.1183
14	2	0.009	1.2200	29.5	3.2391
16	1	-0.003	0.8400	17.0	-0.4284
16	2	0.017	0.8400	17.0	2.4276
17	1	0.076	0.0085	43.0	0.2778
18	1	0.300	0.5300	8.0	12.7200
18	2	0.071	0.5300	8.0	3.0104
19	1	0.075	0.2900	18.0	3.9150
19	2	0.030	0.2900	18.0	1.5660

[1] Activation conditions are: ACT 1 = -S9; ACT 2 = +S9.
[2] Slope of linear portion of dose response curve.
[3] Particle Emission Rate.
[4] Percent of total particle weight extracted.
[5] SAR (SCE/KM) Sample Activity Rate.

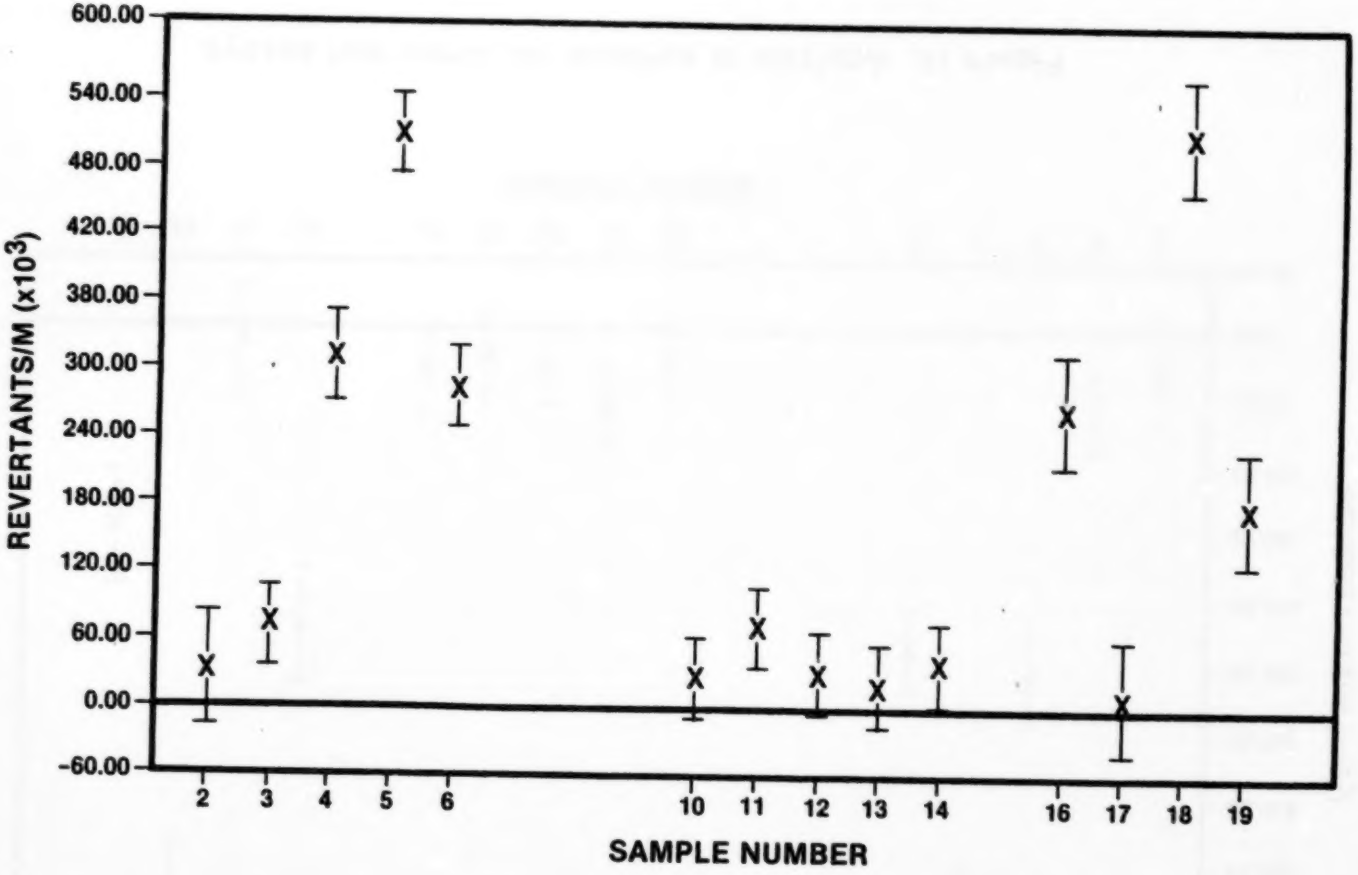


Figure 10. Analysis of variance for Ames test assays.

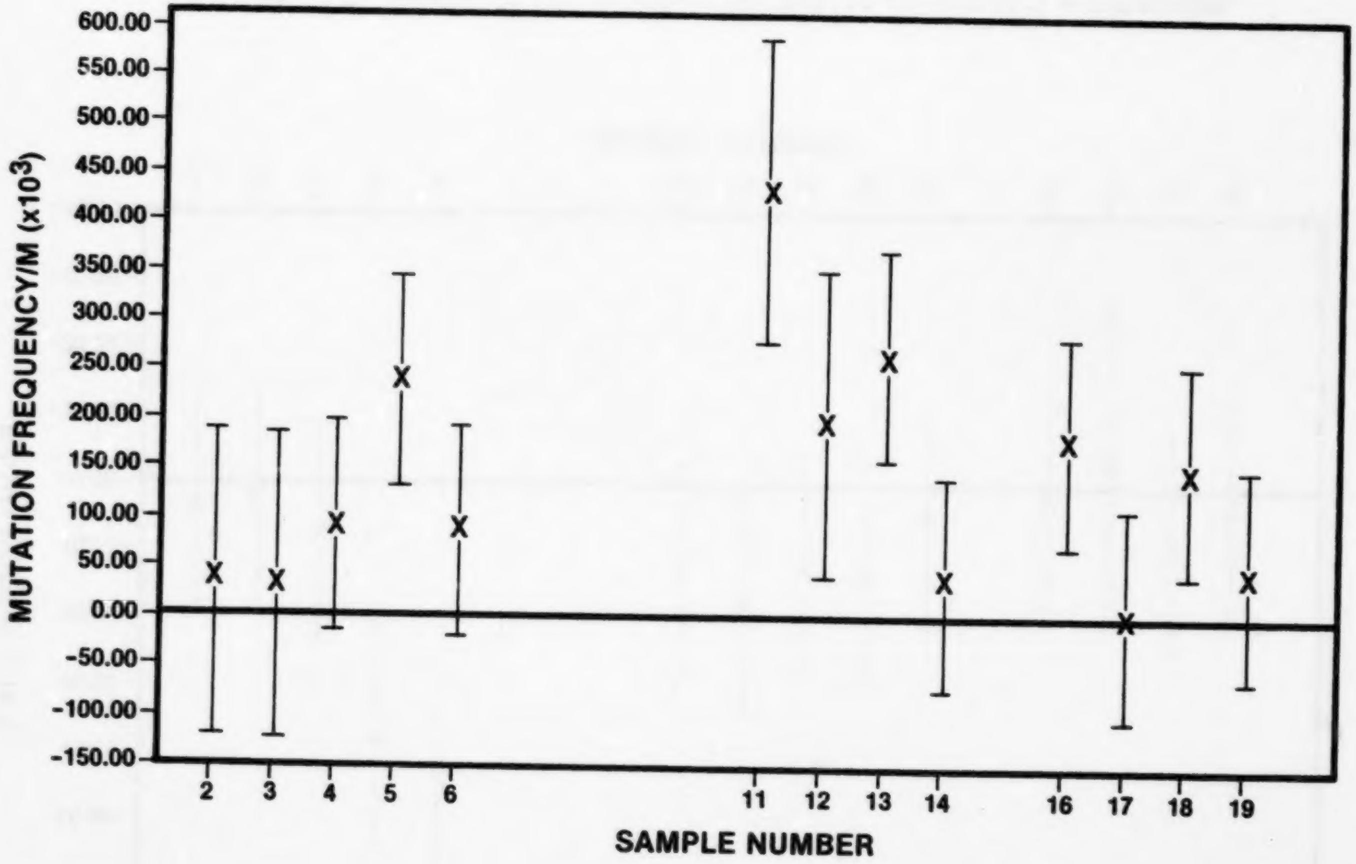


Figure 11. Analysis of variance for mouse lymphoma assays.

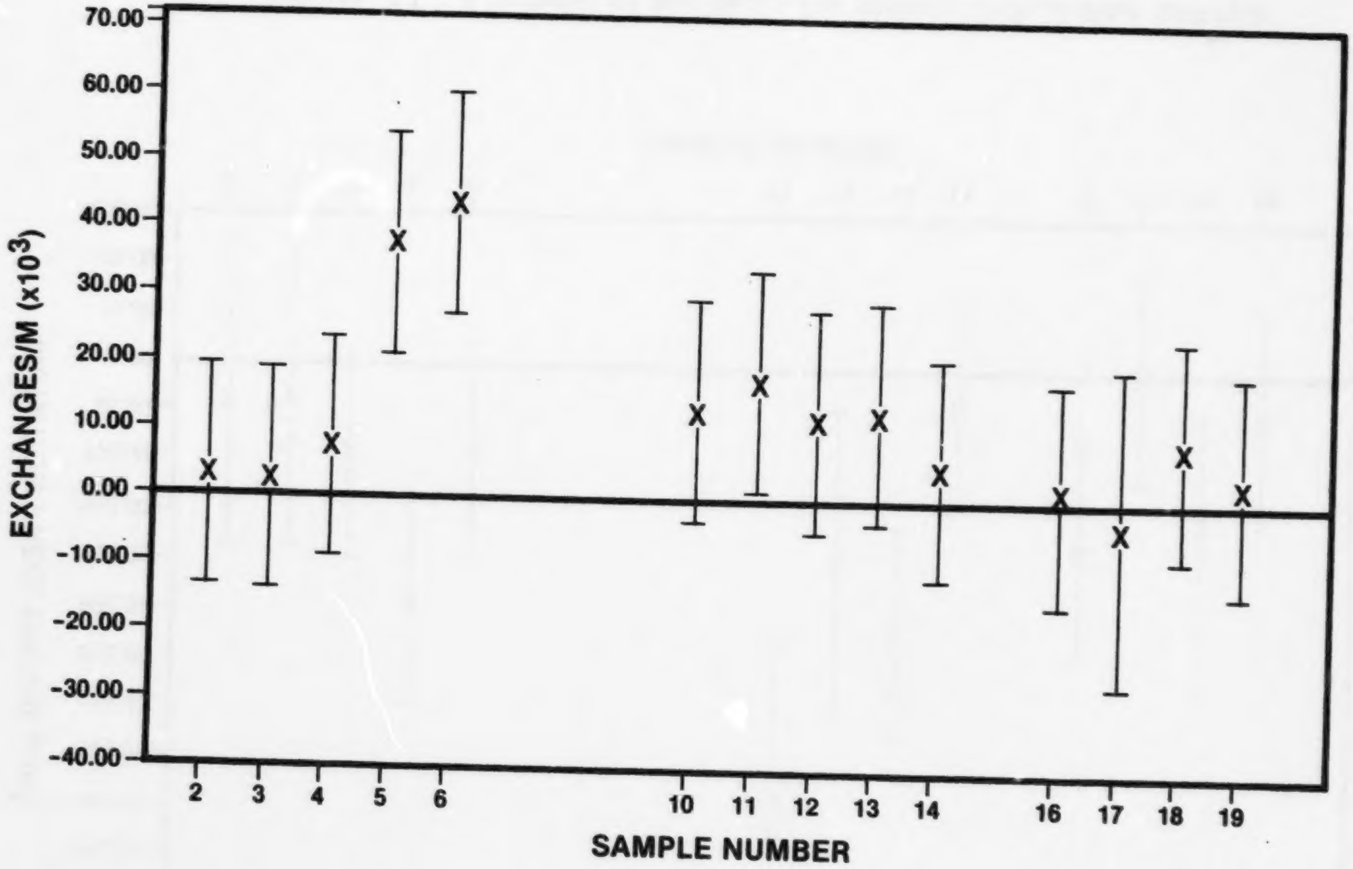


Figure 12. Analysis of variance for sister chromatid exchanges.

Several conclusions can be made based on analysis of even this small set of samples.

1. Comparisons between nonactivated and S9-activated tests indicated that the set of samples studied in S9 had no effect on the responses.
2. The biological activities calculated for the emission samples did not appear to follow similar patterns in the three tests.
3. Among the three tests, the Ames assay was the most discriminating for this set of emission samples. It is likely, however, that greater use of replicate trials in the mammalian cell assays would increase their discriminating properties.
4. As a consequence of the analysis of this pilot data it would appear that replication of sample collection, analysis, and biological testing is essential. Recommendations for replication of these components of the proposed scheme are shown in Figure 13. By performing independent trials, the variability arising from replicating the collection and extraction procedures as well as bioassay techniques are important data components. Claxton and Kohan (1981) showed that there are small but consistent variances resulting from sample preparation differences.

DATA INTERPRETATION

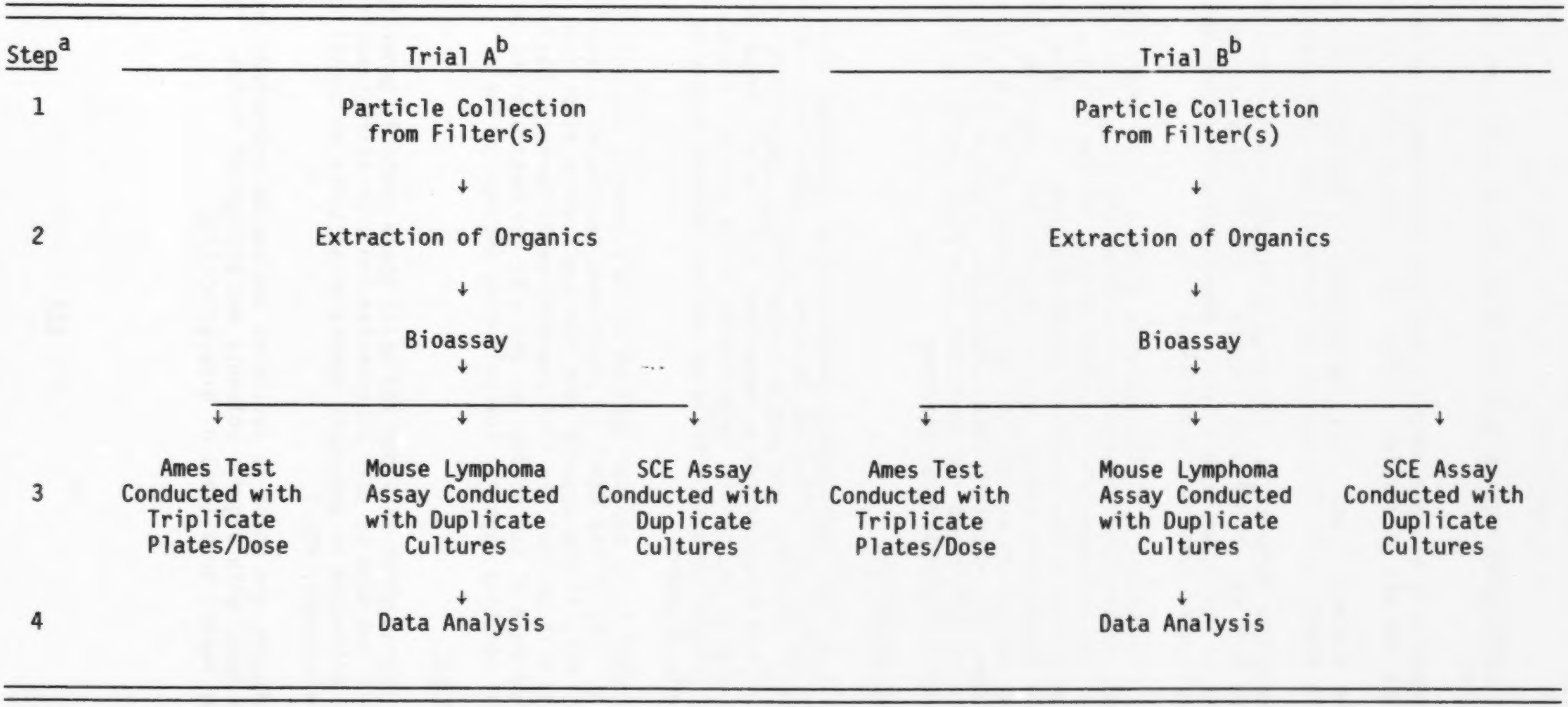
If engine or fuel assessments studies can be conducted under conditions in which certain variables can be carefully controlled (running cycles, collection conditions and sample preparation), comparisons of hazard potential can be generated as described previously. These will be best estimates with reasonably large limits of variation. Standardized engine running conditions and particle collection methods should be defined in a separate document.

As indicated in an earlier section of this report, the ability to quantify hazard or risk on the basis of short-term testing will require continued research but, if one assumes that the qualitative risks from automobile emission will be similar, then comparative differences based on a statistical analysis of the biological activity parameters might be a reasonable means to identify high vs. low polluting engines or fuels.

CONCLUSIONS

1. Mutagens exist in vapor and solid phase probably generated by combustion and mono or poly substituted PAH's or nitroaromatics which can be activated to genotoxic agents by bacteria or animal cells (liver, macrophages, etc.).
2. Mutagens are bound to particles but can be extracted, to various degrees, with organic solvents and biological fluids. Thus, one can expect some level of bioavailability.

FIGURE 13. COMPOSITION AND LOGISTICS
OF AN EMISSION ASSESSMENT


Step[a]
Trial A[b]
Trial B[b]
1
Particle Collection from Filter(s)
Particle Collection from Filter(s)
2
Extraction of Organics
Extraction of Organics
Bioassay
Bioassay
3
Ames Test Conducted with Triplicate Plates/Dose
Mouse Lymphoma Assay Conducted with Duplicate Cultures
SCE Assay Conducted with Duplicate Cultures
Ames Test Conducted with Triplicate Plates/Dose
Mouse Lymphoma Assay Conducted with Duplicate Cultures
SCE Assay Conducted with Duplicate Cultures
4
Data Analysis
Data Analysis

[a] Step 1 - Independent trials for determination of PER
Step 2 - Independent trials to measure percent extractables
Step 3 - Replicate data within independent trials for each of the three bioassays
Step 4 - Data analysis and comparison with other emission samples

[b] Definition of independent trials A and B has not been made. Trials may be different runs on the same day or different runs on two days. The total particulate required per trial is approximately 0.5g.

3. The levels and composition of chemicals found in emissions may be influenced by:

   A. Engine types
   B. Fuel types
   C. Operating conditions
   D. Control devices
   E. Environmental conditions of collection.

4. An efficient screening program to compare quantitative variations in one of the above parameters (by holding all other parameters constant) can be accomplished with a series of short-term *in vitro* tests; however, the assessment cannot be conducted on whole emission collections; rather it requires a standard sample collection and processing protocol for the preparation of organic compounds recovered from the solid phase.

5. A uniform data interpretation scheme has been defined in which the relative biological activity of each bioassay can be quantitatively compared to an existing data base developed from similar engines, fuels, or fuel additives. Samples which show statistically significant deviations from the data base can be interpreted as a signal that an engine, fuel, or additive might warrant further investigation for possible health impact.

The proposed approach should be adequate for making quantitative comparisons of the biological activity of emissions generated by different engines or fuels under standardized running and collection protocols and for identifying responses which fall outside the normal range of engines, fuels, or additives. The results will not be suitable to quantify the absolute health risk of emissions because of the many uncertainties associated with bioavailability and dosimetry of emissions to heterogenous human populations under normal exposure conditions and the lack of formal linkages between the responses in short-term tests and human disorders.

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